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(54) **LHRH analogues useful in stimulating anti-LHRH antibodies and vaccines containing such analogues.**

(57) A conjugate between a nona- or decapeptide of the formula i) or ii):

i) Lys-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, or

ii) Cys-Lys-Trp-Ser-Try-Gly-Leu-Arg-Pro-Gly-NH_2, or

mixtures of peptides i) and ii).

and a protein in provided which, when used per se or when mixed with a suitable adjuvant, yields a vaccine which acts as an immunogen for LHRH and induces a mammal to produce antibodies which react with LHRH. Immunization against the body's LHRH results in lowering of male and female sex hormones including luteinizing hormone so as to prevent conception. Other uses for materials which lessen the effect of LHRH in the body are known in the art.

LHRH ANALOGUES USEFUL IN STIMULATING ANTI-LHRH ANTIBODIES AND VACCINES CONTAINING SUCH ANALOGUES

Background of the Invention

Luteinizing Hormone Releasing Hormone ("LHRH") is secreted by the hypothalamus and carried to the pituitary gland where it stimulates secretion of follicle stimulating hormone and luteinizing hormone which, in turn stimulate ovarian follicle development, the conversion of ovarian follicular to corpus luteum, tubule development in the testicles and production of progesterone and testosterone. Thus, release of LHRH causes ovulation and formation of corpus luteum in females and spermatogenesis in males.

LHRH is a decapeptide of the following structure:

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

wherein, according to convention, the amino group of each amino acid appears to the left and the carboxyl to the right with the hydroxyl of the carboxyl of the terminal Gly being replaced by an NH_2 group. The conventional abbreviations for the amino acids are: Glu (glutamic acid), pGlu (pyroglutamic acid), His (histidine), Trp (tryptophane), Ser (serine), Tyr (tyrosine), Gly (glycine), Leu (leucine), Arg (arginine), Pro (proline), Lys (lysine) and Cys (cysteine). Except for glycine which has no optical center, all amino acids are of the L-configuration unless otherwise indicated. LHRH may be produced as described in U.S. Patents 4,159,980 and 4,213,895.

Analogues of LHRH have been prepared which act as agonists or antagonists of LHRH, i.e., which tend to diminish or accentuate the action of LHRH in the body. Such analogues

are described in U.S. Patents 3,880,825; 3,941,763; 4,034,082; 4,072,668; 4,075,192; 4,143,133; 4,143,136; 4,211,769; 4,234,571; and 4,263,282. These analogues may be administered to the animal or patient in amounts such as 2 to 200 micrograms per kilogram of body weight to yield an immediate effect on the reproductive cycle as described in U.S. Patent 4,010,261. A second type of treatment is the administration to the patient or animal of an LHRH analogue as an antigen, i.e., immunogen, whereby the analogue acts as a vaccine and the host mammal generates antibodies to the analogue which also act against the body's own LHRH. Thus, the analogue's effect will persist after the analogue itself has been metabolized or excreted. This second treatment is described for various LHRH analogues or LHRH itself by A. Arimura et al. in Enocrinology 93:1092-1103 (1973); by H.M. Fraser et al. in the Journal of Endocrinology 63:399406 (1974); by S.L. Jeffcoate et al. in Immunochemistry Vol. 11, p. 75-77 (1974); by I.J. Clarke et al. in the Journal of Endocrinology 78:39-47 (1978); by L. Pique et al. in Immunochemistry Vol. 15 pages 55-60 (1978); by V.C. Stevens et al. in the American Journal of Reproductive Immunology 1:307-314 (1981); and in U.S. Patent 3,963,691.

An object of the present invention is a vaccine containing an immunogen which prevents the function of LHRH when administered to a male or female mammal. At present there are 43 million dogs and 31 million cats in the U.S. and their numbers increase daily. Stray dogs and cats along with wild animals such as skunks and raccoons are known to be major sources of rabies transmission to domestic animals and humans. Surgical removal of reproductive organs, e.g., spaying and castration, is presently a

commonly used method for preventing reproduction in mammals. However, surgery is relatively costly, time consuming and impractical when used with wild or stray animals. A vaccine which immunizes the animal against its own LHRH would prevent conception for extended periods and would be a cost-effective method of population control. A further object is a vaccine form which can be used in dart-guns or drug-containing bullets for the immunization of wild mammals. An object of the invention is a vaccine for population control of a large population of animals such as deer, wild horses and burros and animals kept in zoos.

A further object of the invention is a vaccine containing an immunogen for the treatment of male mammals for the undesired effects of LHRH in such animals. For example, cryptorchidism is a condition where one or both testicles of a male mammal have not descended from the abdomen making castration a difficult surgical procedure. A vaccine which prevents LHRH from transmitting signals to produce male hormones would be, in effect, a "immunological castration" for male mammals and could be used to render cryptorchid stallions docile.

Summary of the Invention

The invention comprises an immunogenic vaccine which contains the nonapeptide Lys-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 or the decapeptide Cys-Lys-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. When administered to a mammal, the vaccine induces in vivo production of antibodies to the host's LHRH whereby the natural secretion of LHRH is neutralized. The vaccine can be used to immunize the mammal against conception or any other conditions which are directly or indirectly influenced by secretion of

LHRH. For example, the vaccine can be used in the treatment of prostate cancer in men.

Detailed Description of the Invention

The vaccine of the invention contains as the immunological agent, a conjugate between a protein and a peptide selected from:

i) Lys-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, or

ii) Cys-Lys-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

or mixtures of i) and ii). The vaccine can be administered as the conjugate per se in solid form as a lyophilized solid or may be micro- or macroencapsulated. Preferably, the vaccine is used as a liquid emulsion, most particularly as a water-in-oil emulsion with the conjugate in the aqueous phase. The emulsion may be described as an adjuvant, the protein as a carrier protein and the peptide as an LHRH analogue.

Peptides i) and ii) are written above using conventional abbreviations where the amino group of each amino acid appears to the left and the carboxyl group to the right. The last 8 amino acids of both compounds are the same and in the same order as the last 8 amino acids of LHRH. The individual amino acids making up peptides i) and ii) above and iii) described below are preferably each of the L-configuration in view of their lower cost compared to the D-configuration. However, the invention comprises peptides wherein each or any of the amino acids are of the D-configuration. Peptides i) and ii) may be obtained from Peninsula Laboratories, Inc. of 611 Taylor Way, Belmont, [illegible] peptide synthesizers.

including Bachem Inc. of 3132 Kashiwa Street, Torrance, CA 90505 and Vega Biochemicals of Tucson, Arizona 85734. Further, they may be prepared by conventional methods known in the art such as by solid phase synthesis using benzhydrylamine resin, protected amino acids, a coupling reagent such as dicyclohexylcarbodiimide (DCC), removal of protecting groups with liquid hydrofluoric acid and purification by counter current distribution, C_{18} column high pressure liquid chromatography and gel chromatography. Such techniques are described in the text by John Stewart and Janice Young entitled "Solid Phase Peptide Synthesis", W.H. Freeman & Co., San Francisco, 1969 and in U.S. Patent 3,941,763. Benzhydrylamine resin may be prepared as described by P. Rivaille et al. in Helvetica Chimica Acta, Vol. 54 pages 2772-2775 (1971). Peptide synthesis may also be accomplished by solid phase synthesis and segment condensation synthesis as described in "The Proteins" Ed. by Hans Neuath et al., Vol 2, 3rd edition, Academic Press, N.Y. (1976) at pages 105-253 written by Frances M. Finn et al. and at pages 257-527 written by Bruce W. Erickson et al., respectively.

The carrier protein used in the invention is preferably one with a molecular weight of at least about 40,000 dalton and more preferably at least about 60,000 dalton. In a particular aspect of the invention, the protein may be of human origin such as to heighten the immune response when the vaccine is administered to an animal while rendering the vaccine less dangerous to a human if it is accidently given to a human. Peptide i) contains alpha- and epsilon-NH_2 groups for conjugation to the carrier protein. Except at very low pH, peptide ii) dimerizes quickly through the SH groups of Cys to the peptide of the following formula iii):

iii)

$SCH_2CH(NH_2)CO$-Lys-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

|

$SCH_2CH(NH_2)CO$-Lys-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

Formation of the dimer makes conjugation through the SH group uncertain. However, the disulfide-bonded dimer iii) may conjugate to the carrier protein through either $-NH_2$ group to provide the advantage of 2 LHRH analogue determinants through one bond between an $-NH_2$ group of the analogue and one -COOH group of the carrier protein. Carrier proteins which may be used in the invention include albumin, such as from bovine, baboon, dog, chicken egg, turkey egg, goat, guinea pig, hamster, human, mouse, pigeon, porcine, rabbit, rat, sheep or other sources, immunoglobulin from such sources or hemocyanin such as from Keyhole Limpets such materials being available from Sigma Chemical of St. Louis, Mo. Keyhole Limpet Hemocyanin (KLH), e.g., as obtained from Cal Biochem of La Jolla, CA, is preferred in view of its high immunogenicity.

Conjugation between the peptide and the carrier protein may be carried out as described by J. H. Kennedy et al. in Clinica Chimica Acta, Vol. 70, pages 1-31 (1976) with conjugating agents such as glutaraldehyde or a water soluble carbodiimide, e.g., 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (ECDI).

The conjugate may be administered per se as a vaccine or preferably micro- or macroencapsulated. Alternatively, the conjugate is provided with an adjuvant for administration to the mammal. This is preferably a water-in-oil emulsion, it being found that an oil-in-water emulsion gives markedly inferior results. Thus, an aqueous solution of the peptide-protein conjugate of the

invention is formulated into a stable water-in-oil emulsion using an oil phase consisting of an oil such as mineral oil and a non-ionic emulsifier. Suitable mineral oils include those having a viscosity of about 8 to 20 centistokes at 40°C, e.g., Drakeol 6 obtained from the Penreco Division of the Pennzoil Co. Nonionic emulsifier include Montanide 888 obtained from Seppic of 70 Champs Elyees, Paris, France. The non-ionic emulsifier may be used with the oil in a weight ration of about 1:6 to 1:12, e.g., about 1:9. For a water-in-oil emulsion, the aqueous phase is slowly added to the oil phase in a homogenizer after which the mixture is emulsified to yield an emulsion having a viscosity of about 200 to 400 centistokes at 40°C. Viscosity measurements may be taken on a Cannon-Ubbelohde Viscometer, available from Cannon Instruments Co. of State College, PA as described in U.S. Patent 2,805,570. The thus-produced vaccine emulsion is stable for at least 1 month at 37°C.

The emulsion vaccine may be administered parenterally to a mammal. Vaccine in the liquid form, e.g., in a water-in-oil emulsion, may be injected by syringe. In the solid form, e.g., lyophilized conjugate, may be used in a ballistic implant or dart gun arrangement as described in U.S. Patents 3,948,263 and 3,982,536 which are incorporated by reference. Such devices are available from BallisiVet Inc. of White Bear Lake, Minnesota. The amount of conjugate to be administered to the mammal to achieve production of anti-LHRH antibodies essentially equivalent to be host's production of LHRH will depend on the degree of conjugation between peptide and protein and the size and species of the host. In general, about 0.2 to 1.0 mg of conjugate per kilogram of body weight is administered and is given twice at a 3 to 6 week interval. Annual booster administrations of the same dose is recommended

recommended for a continued effect. Since the antibody titer will decrease gradually, the effect of the invention vaccine will diminish and is thus reversible, which is an advantage of the invention over prior surgical methods.

The vaccine of the invention, as described above, may be used to treat any condition in man or other mammals which is brought on or aggravated by LHRH. The vaccine is thus an effective contraceptive agent in males and females, an agent to treat sexual hyperactivity in males and females, e.g., for the treatment of cryptorchidism in male mammals such as horses, and the treatment of cancers and other conditions which are stimulated by sexual hormones. For example, cancer of the prostate gland is believed to be advances by male hormones and removal of male gonads or injection of antagonistic female hormones is often used for treatment. The anti-LHRH vaccine of the invention may be used to treat prostate cancer by preventing LHRH from signaling the secretion of male hormones.

Example 1

Preparation of Peptide ii)

Synthesis of peptide ii) was carried out by the solid phase method useing para-metyylbenzhydrylamine resin with the projected amino acids, in order of coupling: Boc-Gly, Boc-Pro, Boc-Arg(Tos), Boc-Leu, Boc-Gly, Boc-Tyr(BrZ), Boc-Ser(OBzl), Boc-Trp, Boc-Lys(CIZ) and Boc-Cys(MBzl). The coupling reagent was DCC (dicyclohexylcarbodiimide), Boc (butyloxycarbonyl) removal by trifluoroacetic acid and neutralization by triethylamine. After the synthesis on a Beckman Model 990 Synthesizer, the peptide was removed from the resin and all protecting groups by liquid HF. Purification was by counter current distribution and gel chromatography

In more detail, the cycle of steps used for the addition of each amino acid is as follows, where washes are for one minute each, unless otherwise stated.

1. Methylene chloride, three times

2. 40% trifluoroacetic acid in methylene chloride, once for two minutes

3. 40% trifluoroacetic acid in methylene chloride, once for 25 minutes

4. Methylene chloride, once

5. Ethanol, once for two minutes

6. Methylene chloride, twice

7. 10% triethylamine in methylene chloride, once for two minutes

8. 10% triethylamine in methylene chloride, once for 10 minutes

9. Methylene chloride, three times

After this series of washes, the appropriate amino acid derivative and dicyclohexylcarbodiimide are added at three-fold excess, and coupling to the growing peptide proceeds for the next two hours. The first amino acid residue is incorporated by coupling Boc-glycine to the benzhydrylamine styrene resin. Serine is added as the benzyl ester and cysteine as the S-methoxybenzyl derivative. Tyrosine is added as Boc-Tyr(Br-Z), arginine as Boc-Ar(os) and lysine as Boc-Lys(Cl-Z). After the peptide has been synthesized, it is given a final deprotection

with trifluroacetic acid and is washed with methanol and dried. The peptide is removed from the dry resin with anhydrous hydrogen fluoride, using anisole to minimize side reactions. This reaction requires about 45 minutes at 0 degrees C. The resin is then dried under vacuum. The peptide-polystyrene mixture is washed with ether and the peptide is extracted with 10% acetic acid. The peptide solution is lyophilized to give the crude peptide. Three grams of crude peptide were applied in two lots to a countercurrent distribution apparatus, using the solvent system described below for peptide i). Each run gave 0.9 grams of partially purified peptide. The 1.8 grams of peptide was applied to a large P-2 column in 10% acetic acid. The peptide was easily soluble in 10% acetic acid and the eluate was collected at 4 drops per second and 15 ml/tube. Tubes 27-35 contained 1.005 grams of pure peptide.

Preparation of Peptide i)

Synthesis of peptide i) was carried out as peptide ii) above with protected amino acids, in order of coupling, Boc-Gly, Boc-Pro, Boc-Arg(Tos), Boc-leu, Boc-Gly, Boc-Tyr(Br-Z), Boc-Ser(OBzl), Boc-Trp and Boc-Lys(Z). Purification was by countercurrent distribution and C_{18} column purification.

In more detail, peptide i) was made by coupling the first amino acid, glycine, as Boc-glycine in BHA resin. From this point the synthesis of the crude peptide was as above, as was the HF cleavage. Three grams of the crude peptide were applied to a counter-current distribution apparatus using the solvent system 4:1:5 of butanol:acetic acid:water. Fractions 88 to 109 contained 0.9 grams of a partially purified peptide. These fractions were pooled and lyophilized. The partially purified peptide was

applied to a C-18 column where the initial solvent was 0.1% acetic acid and the final solvent was 80% acetonitrile containing 0.1% acetic acid. A linear gradient was used with 700 ml of each solvent. Peptide fractions that appeared pure by TLC were pooled and submitted to quality control.

Example 2

Peptide i)-BSA Conjugate

Into 1 ml of 0.85% NaCl solution made with distilled water was dissolved 20 mg of bovine serum albumin (BSA). 10 mg of peptide i) was dissolved in 1 ml of distilled water and slowly added to the BSA solution with constant mixing. 100 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (ECDI) was dissolved in 0.5 ml of distilled water and slowly added to the BSA solution with constant mixing. The mixture is then incubated for 6 hours at room temperature with constant stirring in a shaker. The mixture was then dialyzed against 2 to 3 liters of phosphate buffer saline (PBS) for about 16 to 24 hours at 5°C. The dialyzing process was repeated twice. The dialyzed conjugate was then filter sterilized and assayed for the degree of conjugation.

Example 3

Peptide i)-HSA Conjugate

The procedure of Example 2 was repeated substituting human serum albumin (HSA) for BSA to obtain a peptide i)-HSA conjugate.

Example 4

Peptide i)-KLH Conjugate

An aqueous solution of about 1.5 to 2% Key Hole Limpet (KLH) was dialyzed against a 0.85% NaCl saline solution for about 36 to 48 hours with two changes of saline solution. The KLH solution protein concentration was then determined by the Biuret Reaction Method using a BSA standard. The Biuret method is described by A. G. Gornall et al. in the Journal of Biological Chemistry, Vol. 177, page 751 (1949). As determined by the Biuret method, 20 mg of KLH was then conjugated to 10 mg of peptide i) as described in Example 2 to yield a peptide i)-KLH conjugate.

Example 5

Peptide ii)-HSA Conjugate

A conjugate or peptide ii) and HSA was prepared by the method described in Example 3 substituting peptide ii) for peptide i).

Example 6

Peptide ii)-KLH Conjugate

The procedure of Example 4 was repeated substituting peptide ii) for peptide i) to obtain a conjugate between peptide ii) and KLH.

Example 7

Peptide ii)-HgG Conjugate

Twenty mg of human gamma globulin (HgG) was dissolved in a mixture of 2 ml of PBS and about 0.25 ml of dimethylformamide into which had been dissolved 5 mg of m-maleimidobenzoyl-N-hydroxysuccinimide (MBS). The thus-produced mixture was allowed to react at room temperature for 25 minutes. The activated HgG was then separated from unreacted materials by adding PBS and subjecting the mixture to gel filtration on SEPHADEX® G 25 gel available from Sigma Chemical Co. of St. Louis, MO. To the activated HgG in PBS was added 10 mg of peptide ii) which was then incubated for 2 hours at room temperature. The mixture was dialyzed as in Example 2, filter sterilized and assayed for the degree of conguation.

EXAMPLE 8

Determination of Degree of Conjugation

The degree of conjugation of peptide i) or ii) to the carrier protein in Examples 2-7 was estimated by the Biuret method. In this procedure, the total weight of protein in the unconjugated protein is determined along with the protein weight in the conjugate, after dialyzing the conjugate to remove any unconjugated peptide i) or ii). The difference in protein weight is the conjugated peptide and from this, the degree of conjugation of peptide to carrier protein can be determined.

Using the biuret technique, it was found that the degree of conjugation of the conjugates of Examples 2-7 was about 10 to 40 peptides per 100,000 dalton of molecular weight of the carrier protein.

Examples 9-14

Adjuvant Vaccines

The conjugates produced in Examples 2-7, respectively, were individually diluted with phosphate buffer saline (PBS) to make 5 to 15 mg/ml solutions. Thiomersal (1 part per 10,000 by volume) was added as a preservative and the material was sterilized either by filter sterilization, e.g., through a 0.2 µm disc, or by gamma irradiation. This constitutes the aqueous phase of the water-in-oil adjuvant vaccine. The oil phase was made by mixing 1 part of the nonionic emulsifer Montanide 888 with 9 parts of Drakeol 6 light white mineral oil followed by filter sterilization. The sterile oil phase is placed in the proper size emulsifying or homogenizing flask equipped with side tubelettes. A Virtis homogenizer Model 23 or 45 made by Virtis of Gardiner, N.Y. 12525 is started at low speed and an equal amount of the aqueous phase was added slowly. After the entire aqueous phase was added, the mixture was emulsified at a higher speed until an effective water in-oil emulsion was made.

The stability of the individual emulsions was determined by centrifuging a sample of the emulsion at 10,000 to 12,000 G for 6 minutes at which no more than 5% separation was detected. The viscosity of the preparation was typically between 260 to 300 centistokes per second. The emulsions were found to be stable for more than one month at 37°C.

EXAMPLE 15

Vaccine Efficacy -- Rats

The vaccines produced in Examples 9-14 from the conjugate produced in Examples 2-7, respectively, were tested for blockage of the effects of LHRH in rats.

Young rats were inoculated intramuscularly with 1.0 ml, 0.5 ml and/or 0.2 ml of the vaccines produced in Examples 9-14 followed by a booster injection of the same dose, 3-4 weeks after the first injection.

Serum titer for antibody against LHRH was determined by the ELISA test as described by A. Voller et al. in the Bulletin of the World Health Organization, Vol. 53, pages 55-65 (1976) and in the "Manual of Clinical Immunology", Chapter 69, pages 506-512, American Society of Microbiology (1976). Microelisa plates were coated with synthetic LHRH. Antibody titers were determined at 4-6 weeks intervals.

In addition, the effectiveness of the vaccine was determined by observing the atrophy of the testicles in the case of males and the uterus and ovaries in the females. The results are shown in the following Table I.

TABLE I

RESULTS OF ANTIFERTILITY VACCINE

Example	Conjugate	Dose	Antibody Titer Range	Gonadal Atrophy*
9	i)-BSA	1.0 ml	640 - 6400	7/7**
10	i)-HSA	1.0 ml	<50 - 6400	3/4
10	i)-HSA	0.5 ml	800 - 6400	3/4
10	i)-HSA	0.2 ml	50 - 6400	0/5
11	i)-KLH	1.0 ml	>6400	4/4
11	i)-KLH	0.5 ml	800 - 6400	3/3
11	i)-KLH	0.2 ml	50 - 6400	5/5
12	ii)-HSA	1.0 ml	>6400	5/5
12	ii)-HSA	0.5 ml	200 - >6400	2/5
12	ii)-HSA	0.2 ml	1600 - >6400	3/5
13	ii)-KLH	1.0 ml	400 - >6400	4/4
13	ii)-KLH	0.5 ml	800 - >6400	4/4
13	ii)-KLH	0.2 ml	<50 - 6400	4/5
14	ii)-HgG	0.5 ml	800 - 6400	4/5

*Atrophy is considered to be positive if the size has diminished to 75% or less of the control group.

**Number of vaccinated animals with positive atrophy/number vaccinated.

From the above data, the peptide i)-KLH vaccine is considered to consitute the preferred embodiment of the invention.

Example 16

Vaccine Efficacy -- Cats

The vaccine produced in Example 11 was tested for efficacy in cats. Five female and three male cats were each given a 0.5 ml injection of the peptide i)-KLH vaccine with a second 0.5 ml injection 4 weeks after the first. Immediately before the second injection, the anti-LHRH antibody titer range for the cats was found to be 800-1600 and 4 weeks after the second injection, the range was 12,800-51,200.

The female cats did not come into heat 18 months after the first injection even after being exposed to a normal male cat.

The male cats all had gonadal atrophy according to the atrophy definition in Example 15 for at least one year after the first injections. One male cat showed gonadal reversion to a normal size 18 months after the first injection.

Example 17

Vaccine Efficacy -- Horse

The vaccine produced in Example 10 was tested for efficacy against cryptorchidism in horses. A cryptorchid stallion was found to have a serum testosterone level of 0.7 nanograms per ml and an anti-LHRH antibody titer of 10 as determined by the Elisa test using LHRH as the antigen. The animal was injected with 3 ml of the peptide i)-HSA vaccine produced in Example 10. A second injection of 3 ml of the vaccine was made 4 weeks after the first. Just prior to the second injection, the animal's serum

testosterone level was found to be 0.09 nanograms per ml with an antibody titer of 2560. Four weeks after the second injection, these figures were found to be 0.05 and 20,480, respectively.

CLAIMS 0181236

1. A peptide select d from the group consisting of:

(i) Lys-Trp-Ser-Tyr-Gly-L u-Arg-Pro-Gly-NH_2;

(ii) Cys-Lys-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Nh_2; and

(iii) $SCH_2CH(NH_2)CO$-Lys-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
|
$SCH_2CH(NH_2)CO$-Lys-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2.

2. The peptide (i) of claim 1.

3. The peptide (ii) of claim 1.

4. A mixture of peptides (i) and (ii) of claim 1.

5. A conjugate between a protein and a peptide of any one of claims 1 to 3 or the mixture of peptides of claim 4.

6. The conjugate of claim 5, wherein the protein has a molecular weight of at least 40,000 Daltons.

7. The conjugate of claim 5 or claim 6, wherein said conjugate comprises from 10 to 40 peptide molecules per 100,000 Daltons molecular weight of the protein.

8. An immunogen vaccine for inducing in a mammal production of antibodies against LHRH, comprising a water in oil emulsion, the water phase comprising a conjugate of any one of claims 5 to 7.

9. The vaccine of claim 8, wherein the oil phase comprises a non-ionic emulsifier.

10. The product of any one of claims 5 to 9 for use in inducing in a mammal production of antibodies against LHRH, for use in preventing ovulation in a mammal or for use in treating a mammal for cryptorchidism.

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(54) Procédé d'immunoneutralisation anti-LHRM des animaux domestiques mâles non castrés et peptide pour cela.

(57) Le procédé pour améliorer les qualités organoleptiques, en particulier l'odeur, la sapidité et la tendreté, de la viande des animaux domestiques mâles non castrés, comprend, peu avant l'abattage de l'animal concerné, la suppression de l'action des stéroïdes androgènes et non androgènes, par immunoneutralisation active ou passive anti-LHRH, tout en maintenant pratiquement jusqu'à l'abattage les avantages liés au caractère mâle de l'animal. Les vaccins comprennent comme peptide la LHRH naturelle ou un peptide de formule Trp - Ser - Tyr - Gly - Leu - Arg - Pro - Gly - NH_2, couplé à une protéine porteuse immunogène.

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La présente invention concerne un procédé pour améliorer les qualités organ leptiques, en particulier l'odeur, la sapidité et la tendreté, de la viande des animaux domestiques mâles non castrés, notamment des bovins, des ovins et des porcins mâles.

L'invention concerne aussi des vaccins utilisabl s dans ce procédé, un nouveau p ptid pour la réalisation de tels vaccins et un ensemble de vaccination y relatif.

Les avantages de l'utilisation du mâle entier sur le mâle castré dans l'engraissement des animaux domestiques destinés à la production de viande ont été soulignés depuis plusieurs décennies par les zootechniciens. Ils concernent un taux de croissance plus élevé, surtout chez les bovins et les ovins, une meilleure utilisation de la ration alimentaire et une carcasse plus maigre mais plus fournie en masse musculaire chez toutes les espèces domestiques (S.C. SEIDEMAN et al. J., of Animal Science, 1982, 55 (4) 826-840 et M. BONNEAU, INRA Prod. Anim., 1988, 1 (2) 133-140).

Les inconvénients principaux de cette utilisation du mâle entier, rappelés dans les revues citées ci-dessus, concernent l'odeur et la sapidité désagréables chez les porcins et ovins mâles, la moindre tendreté de la viande des bovins et ovins mâles entiers et justifient les pratiques actuelles de la castration chirurgicale.

En effet, si les stéroïdes androgènes dont l'androsténediol, l'androsténedione et la testostérone sont les éléments déterminants des avantages attendus dans toutes les espèces domestiques pour une croissance plus rapide et une meilleure utilisation de la ration alimentaire, ils sont rendus responsables d'une moindre tendreté de la viande des bovins et ovins mâles entiers. Les stéroïdes non androgènes ou les dérivés des 16-androstènes dont la 5 α-androsténone (5α-androstèn-16 one-3), chez le porc mâle, sont responsables en partie de l'odeur et de la sapidité désagréables de la viande d'un certain nombre de porcins mâles entiers dès la puberté, lesquelles déprécient la viande et sont un obstacle à sa commercialisation à l'état frais.

Le scatole, produit dérivé du tryptophane et produit par la flore microbienne intestinale, est un composé responsable en partie de l'odeur et de la sapidité désagréables de la viande du porc mâle entier. Sa production dépend de facteurs de l'environnement, de la nutrition, de la race. Son accumulation dans le tissu adipeux est plus importante chez le verrat et serait liée aux sécrétions des stéroïdes sexuels gonadiques.

A titre expérimental, on a déjà essayé de diminuer ou de supprimer le développement du caractère mâle chez le jeune animal ou la sécrétion d'hormones testiculaires, notamment des stéroïdes testiculaires, par immunoneutralisation active ou passive contre ceux-ci ou contre les hormones intervenant dans leur sécrétion, notamment l'hormone lutéinisante ou LH (Luteinizing Hormone) et l'hormone gonadolibérine (GnRH) encore appelée Luteinizing Hormone Releasing Hormone (LHRH). Des essais ont été conduits sur le porc pour abaisser le taux tissulaire de la 5 α-androsténone, du groupe des 16-androstènes, par l'immunisation active dirigée contre ce composé (E.D. WILLIAMSON et al., Liverstock Production Science, 1985, 12, 251-264) ou par l'immunisation passive contre ce même composé (R. CLAUS, Immunization with Hormones in Reproduction Research, ed. Nieschlag, 1975). La suppression ou la diminution de la sécrétion des stéroïdes testiculaires peut être recherchée par l'immunoneutralisation de l'hormone gonadotrope LH, spécifique de l'espèce considérée (R.E. FALVO et al., J. Anim. Science, 1986, 63, 986-994) ou par l'immunoneutralisation anti-LHRH de la LHRH endogène. Seule l'immunisation active anti-LHRH ,a été préconisée par différents auteurs. Chez le porc, l'abaissement de l'α-androsténone a été obtenu par cette méthode (A. CARATY et M. BONNEAU, C.R. Acad. Sci. Paris 1986, 303, Série III (16) 673-676 ; R.E. FALVO et al., J. Anim. Sci., 1986, 63, 986-994).

Chez le mouton, B.D. SCHANBACHER (Am. J.Physiol., 1982, 242, E201-E205) préconise l'immunisation anti-LHRH pour retarder le développement testiculaire et produire un effet de castration chez les agneaux mâles. Chez les bovins, P.S. ROBERTSON (Vet. Rec., 1979, 105, 516-517) décrit une castration immunologique anti-LHRH.

Les essais d'immunoneutralisation anti-LHRH décrits sur les animaux de laboratoire (ARIMURA et al. Endocrinology, 1973, 93, 1092-1103 ; FRASER H.M. et al., J. Endocr. 1974, 63, 399-406 ; MAKINO T. et al. Contraception, 1973, 8 (2), 133-145 ; CARELLI C. et al., Proc. Natl. Acad. Sci., USA, 1982, 79, 5392-5395) et sur plusieurs espèces domestiques (JEFFCOATE et al., Theriogenology, 1978, 10(4), 323-335 ; ROBERTSON I.S. et al., Veterinary Record.,1979, 105, 556 ; SCHANBACHER B.D. Am. J. Physiol., 1982, 242, E201-E205) ont montré qu'il est possible d'obtenir l'arrêt de la sécrétion de la testostérone, l'involution pondérale des testicules et de ses glandes annexes, l'arrêt de la spermatogénèse et, sur le plan du comportement, la disparition de la libido.

Ces travaux ont conduit à suggérer le recours à une immunoneutralisation, notamment anti-LHRH, précoce pour remplacer la traditionn ll castration chirurgicale à des fins d'élevage.

Dans le brevet US n° 4.556.555, il est ainsi décrit une méthode d'immunisation passive d'animaux avant l ur puberté, à l'aide d'un antisérum contenant des anticorps dirigés contre la gonadotropine.

La demande de brevet internationale WO 90/11298 décrit un procédé d'immunisation anti-LHRH à la naissanc à l'aide de 2 séqu nces de LHRH en tandem couplées à une protéine port use, pour améliorer la qualité de la viande chez le porc.

La demande de brevet internationale WO 88/00056 décrit une méthode de castration immunologique anti-LHRH destinée à améliorer le comportement social et sexuel des animaux mâles en remplacement de la castration chirurgicale qui affecte le taux de croissance. Les taureaux sont vaccinés à l'âge de 8 à 40 semaines et reçoivent ensuite plusieurs rappels.

Un vaccin anti-LHRH vendu sous la marque VAXSTRATE par la société australienne WEBSTERS est utilisé chez la vache.

R.E. Falvo et al. (J. Anim. Sci. 1986, 63 : 986-994) ont immunisé plusieurs groupes de verrats à l'aide de conjugués LHRH-séroglobuline humaine en adjuvant complet de Freund ou avec le muramylpeptide comme adjuvant. Les auteurs ont observé, après vaccination et plusieurs rappels, des titres élevés d'anticorps anti-LHRH, mais avec la nécessité de pratiquer des rappels répétés pour maintenir le titre élevé en anticorps.

I.S. Robertson décrit une méthode d'immunisation avec LHRH conjuguée à l'anatoxine tétanique ou à la thyroglobuline et suggère que l'approche immunologique autoriserait une castration tardive avec les avantages que l'on peut en attendre sur le plan de la croissance pondérale. Il conclut cependant que des efforts sont encore à faire pour arriver à une méthode de castration utilisable dans la pratique, que ce soit au niveau de la méthode elle-même ou de l'adjuvant, l'adjuvant de Freund étant proscrit en pratique.

Enfin, A. Caraty et M. Bonneau (C.R. Acad. Sc. Paris, t. 303, Série III, n°16, 1986) ont pratiqué une immunisation anti-LHRH chez le porc mâle. Les auteurs suggèrent que le blocage de la production de steroïdes, 2 à 3 semaines avant l'abattage, permettrait d'exploiter les potentialités élevées de ce type d'animal pour la production de viande en évitant les problèmes posés par l'accumulation d'androstérone dans le tissu adipeux. Ils concluent cependant que d'importants progrès restent à accomplir dans les techniques d'immunisation avant qu'il ne soit possible de proposer l'immunisation active anti-LHRH comme technique utilisable en élevage porcin.

Par ailleurs, l'immunoneutralisation tardive pose dans la pratique le problème important de l'innocuité du traitement et notamment des réactions locales engendrées par les vaccins, en particulier les vaccins huileux, avec les risques de rejet ou de déclassement de la viande qui en résultent.

L'amélioration des qualités organoleptiques chez les bovins et les ovins n'a pas été suggérée.

La déposante a justement trouvé un procédé applicable industriellement permettant d'améliorer les propriétés organoleptiques de la viande des animaux, procédé dans lequel, peu avant l'abattage de l'animal, on supprime sensiblement l'action des stéroïdes androgènes et non androgènes, par immunoneutralisation active ou passive anti-LHRH, tout en maintenant pratiquement jusqu'à l'abattage les avantages dus au caractère mâle de l'animal.

Selon un premier mode de réalisation préféré de ce procédé, on administre à l'animal un vaccin anti-LHRH, de préférence en émulsion, de préférence pendant ou avant la phase d'engraissement de l'animal, puis, peu avant l'abattage de l'animal, on administre à nouveau un vaccin anti-LHRH. On peut procéder en deux administrations distinctes ou par le biais d'un procédé à libération contrôlée.

Chez le porc, il est particulièrement avantageux d'administrer, avant l'abattage, le vaccin anti-LHRH avec un adjuvant de type aqueux, notamment gel d'hydroxyde d'aluminium et/ou saponine.

Cette administration est effectuée de préférence 15 à 21 jours avant l'abattage.

Au contraire, chez le bovin, et éventuellement chez l'ovin, l'administration précédant l'abattage est faite de préférence avec un adjuvant en émulsion, et de préférence 1 à 2 mois avant l'abattage. Cette administration est effectuée de préférence au moins 4 semaines, et de préférence plusieurs mois, après la première administration.

Dans tous les cas, on préfère, pour le vaccin en émulsion, destiné à la première administration et, chez le bovin, à la deuxième administration, que le vaccin se présente sous forme d'émulsion eau-dans-l'huile. Cependant, d'autres formes d'émulsion sont envisageables.

Ce vaccin, de préférence du type en émulsion, est conçu, selon l'invention, pour l'induction d'une première réponse immunitaire de faible intensité, sans effet notable, ou même mesurable, sur la sécrétion des stéroïdes gonadiques. La formulation en émulsion est préférée, mais les autres formulations sont utilisables dès lors qu'elles produisent ce même effet.

L'administration qui précède l'abattage est faite avec un vaccin formulé pour produire à ce moment la suppression ou l'abaissement significatif de la sécrétion des stéroïdes sans réaction locale ou générale adverse, susceptible de nuire à l'apparence ou à la qualité de la viande.

De façon préférée notamment pour le porc, le conjugué, en solution aqueuse, est mis sous les deux formulations suivantes : la première en émulsion eau-dans-l'huile, stable, faite d'un mélange d'huiles minérales animales ou végétales hautement purifiées et de tensioactifs non ioniques pour l'induction d'une réponse immunitaire de faible intensité, sans effet mesurable sur la sécrétion des stéroïdes,gonadiques, la seconde, non émulsionnée, avec gel d'hydroxyde d'aluminium et saponine, déclenchant une réaction immunitaire rapide et intense se traduisant par la production d'anticorps anti-LHRH neutralisants, suffisante pour entraîner la dimi-

nution ou la suppression des stéroïdes gonadiques t la diminution du transport associé d scatole d'origine intestinale.

L'émulsion utilisée est, à la différence de celle qui est obt nu à l'aide d l'adjuvant complet u incomplet de Freund, un émulsion stabl permettant de préparer un vaccin prêt à l'emploi. La réacti n inflammatoire cutanée reste très faible et localisé aux points d'administration des deux formulations vaccinales et s traduit sous la forme de papules bien circonscrites à l'examen externe. Son développement interne reste limité au derme superficiel. Elle disparaît sans laisser de granulome apparent au moment de l'abattage des animaux.

Selon un autre mode de réalisation de ce procédé, on administre à l'animal, quelques jours avant l'abattage, notamment 5 à 15 jours avant, du sérum ou du plasma hyperimmun anti-LHRH ou encore des anticorps monoclonaux anti-LHRH.

L'immunisation passive anti-MHRH entrainant la diminution voire la suppression de la sécrétion des stéroïdes androgènes et non androgènes a été obtenue par l'administration intramusculaire de plasma hyperimmun équin. Portée à un niveau suffisant, mesuré par le titre en anticorps LHRH du sérum de l'animal receveur, elle entraîne la diminution de la testostérone plasmatique dès le 3ème jour; maintenue à ce même niveau les 12 jours suivants, elle est suffisante pour entraîner l' abaissement de l'androstérone tissulaire au-dessous de 0,50 microgramme/g, valeur pour laquelle l'odeur désagréable et la sapidité particulière de la viande du porc mâle ne seraient plus perçues par le consommateur. Cette méthode d'immunisation passive a montré que le maintien pendant 12 jours de la diminution significative de la testostérone est suffisant pour abaisser la concentration d'androstérone tissulaire au-dessous du seuil fixé. Cette immunisation passive peut être envisagée par l'emploi d'anticorps monoclonaux anti-LHRH secrétés par les hybridomes ou hétérohybridomes porcins.

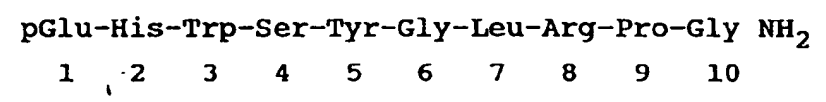
Le mode d'administration de ces formulations est de préférence transcutané, notamment à l'aide d'un appareil d'injection sans aiguille, par jet sous pression, notamment selon la demande de brevet FR-A-2.652.257.

Le procédé selon l'invention présente l'avantage important de présenter une parfaite innocuité, notamment de ne pas induire des réactions locales susceptibles d'entraîner le déclassement de la viande.

La réaction inflammatoire cutanée reste localisée aux points d'administration des deux formulations vaccinales et se traduit sous la forme de papules bien circonscrites à l'examen externe. Son développement interne reste limité au derme superficiel. Elle disparaît sans laisser de granulome apparent au moment de l'abattage des animaux. La réaction inflammatoire, limitée dans le temps et aux points d'administration, traduit la tolérance aux deux formulations vaccinales et est obtenue par l'administration transcutanée de celles-ci, effectuée à l'aide d'un injecteur sans aiguille.

L'immunisation anti-LHRH nécessite de conjuguer le peptide LHRH ou un fragment du peptide LHRH, non immunogène dans les conditions économiques de leur emploi, à une protéine immunogène, dite porteuse, par une liaison covalente.

La LHRH ou GnRH, qu'elle soit naturelle ou de synthèse, est composée de 10 acides aminés, numérotés de 1 à 10 en allant de la terminaison amino-terminale à la terminaison carboxy-terminale suivant la formule suivante :



Ces symboles, par convention, représentent : pGlu, acide pyroglutamique ; His ; histidine ; Trp, tryptophane ; Ser, sérine ; Tyr, tyrosine ; Gly, glycine ; Leu, leucine ; Arg, arginine ; Pro, proline.

Les conjugués immunogènes anti-LHRH, décrits par les différents auteurs peuvent être réalisés en ce qui concerne l'haptène avec :

a) la LHRH totale ou modifiée en une ou plusieurs de ses parties pour obtenir la conjugaison amino-terminale, carboxy-terminale ou intermédiaire souhaitée,

b) avec l'un de ses fragments peptidiques composés de 5 à 7 acides aminés modifiés ou non, pour obtenir la conjugaison aminoterminal , carboxyterminale ou intermédiaire souhaitée,

c) avec un agoniste p rtant un acide aminé substitué, le plus couramment en 6, pour obtenir une conjugaison intermédiaire.

En ce qui concern la protéine porteuse, la sérumalbumin bovine, la sérumalbumine humaine, la thyroglobuline, l'ovalbumin , les globulines humaine ou équine ont été utilisées.

Ainsi, la demande de brevet européen EP-A-181 236 décrit des conjugués immunogènes comprend un nonapeptide ou un décapeptide incluant une séqu nce, correspondant aux 8 derniers acides aminés de la molécule LHRH, à laquelle est ajouté un lysin ou une séquence cystéine-lysin du côté aminot rminal.

D'autre part, la demande de brevet WO 88/05308 divulgue des conjugués constitués à l'aide de fragments

de 5, 6 ou 7 acides aminés contigus de la molécule naturelle, dans lesquels chaqu fragment inclut l'acide pyroglutamique N-terminal ou le glycinamide carboxyt rminal et auxquels un acide aminé ou une séquence d'acides aminés additionnels peuvent être ajoutés à l' xtrémité liée à la protéine immunogène.

Les agents d conjugaison utilisés peuvent être classés en trois grandes catégori s : les agents d'activation, les agents homobifonctionnels, les agents hétérobifonctionnels. Alors que, pour les agents d'activation, la liaison entre les deux molécules se fait entre deux fonctions déjà présentes, pour les autres, la liaison se fait par l'intermédiaire d'un résidu hydrocarboné appelé ligand.

Parmi les agents d'activation, on peut citer l'acide periodique employé pour oxyder les résidus oligosaccharidiques des glycoprotéines en aldéhydes, sur lesquels réagiront ultérieurement les groupements amine de l'autre molécule entrant dans le conjugué.

Les carbodiimides sont des agents d'activation largement employés pour le couplage d'antigènes sur les protéines et, parmi eux, le plus utilisé est sans doute le chlorhydrate de N-éthyl-N'-(diméthylamino-3 propyl) carbodiimide (EDC) qui permet d'effectuer la réaction en milieu aqueux. Leur action conduit à la formation d'une liaison amide entre un groupement carboxyle d'une protéine, activé de façon intermédiaire sous la forme d'une O-alcoylisourée, et un groupement amine porté par une autre molécule. Leur avantage réside dans leur simplicité d'utilisation.

Les agents homobifonctionnels sont des molécules qui possèdent deux groupements réactifs identiques séparés par une chaîne hydrocarbonée. Parmi eux, citons le glutaraldéhyde, qui réagit sur deux groupements amine primaires, les bisisothiocyanates d'alkyle ou d'aryle, qui réagissent sur les amines primaires et les thiols, la benzidine bisdiazotée, qui copule avec les résidus aromatiques de la tyrosine. Mentionnons pour mémoire les bismaléimides et les bisamidinates. L'inconvénient majeur des agents homobifonctionnels est de mal maîtriser la nature des conjugués formés car ces agents peuvent réagir sur deux molécules de même nature et conduire à la formation d'oligomères ou de polymères.

Pour y remédier, des chimistes ont introduit les agents hétérobifonctionnels, dans lesquels les deux groupements ont des spécificités différentes. Dans le cas général, l'un de ces groupements est un ester du N-hydroxysuccinimide qui, dans des conditions douces, réagit sur les groupements amine libres des protéines pour donner d'une part le N-hydroxysuccinimide et d'autre part la protéine portant pa une liaison covalente amide l'agent de couplage sur lequel se trouve la 2ème fonction. Assez généralement, celle-ci peut réagir sur les thiols apportés par la molécule à coupler, ces thiols étant soit initialement présents dans la molécule sous forme de résidus cystéine (ceux-ci pouvant être constitutifs ou, dans le cas de peptides, introduits intentionnellement lors de la synthèse), soit apportés par des agents tels que l'imino-2 thiolane ou le N-((pyridyl-2)dithio-3 propanoyloxy) succinimide (SPDP), après réduction.

Parmi les possibilités énoncées ci-dessus, on préfère utiliser la LHRH totale. En ce cas, la LHRH naturelle est préférée aux agonistes tels que la (D-Lys^6)-LHRH par la comparaison de l'activité immunogène des conjugués préparés avec ces deux peptides.

Le carbodiimide est préféré au glutaraldéhyde comme agent de conjugaison de la LHRH de forme naturelle sur l'alpha-globuline.

L'alpha-globuline humaine ou équine, fraction IV-1 ou IV-4, est préférée à la sérumalbumine humaine ou bovine.

De préférence, les vaccins comprennent un même principe actif, comprenant préférentiellement un conjugué alpha-globuline-LHRH ; la LHRH est de préférence sous forme naturelle et l'alpha-globuline d'origine humaine ou équine, notamment fractions IV-1 et/ou IV-4. Le conjugué est de préférence obtenu par addition sur 1 volume de mélange alpha-globuline et LHRH en solution de 2 à 20 mg/ml dans NaCl 0,9 % de 0,5 à 2 volumes de solution de chlorhydrate de-N-éthyl-N'-(dimétylamino-3-propyl)carbodiimide (EDC) en solution à 2,5 % dans NaCl 0,9 %. Après agitation, le mélange est laissé une nuit, puis purifié par chromatographie de perméation sur gel.

En ce qui concerne la protéine porteuse, on peut utiliser les sérumalbumines, notamment bovine ou humaine, la thyroglobuline, l'ovalbumine, les globulines humaine ou équine, les anatoxines, notamment l'anatoxine tétanique.

La prédominance de la répons immunitaire d s porcs mâles à la fraction carboxyterminale du peptide LHRH conjugué par le carbodiimide ou de son agoniste [D-Lys^6]-LHRH conjugué par le SPDP sur l'alpha-globuline qui a été observée a conduit à la définition d'un conjugué immunogène anti-LHRH utilisant un peptide avantageux présentant la terminaison carboxy-terminale de la LHRH.

Par conséquent, selon un deuxième mode de réalisation préféré de l'invention, la déposant a trouvé qu'il était très avantageux d'utiliser un nouv au peptide comprenant les 8 derniers acides aminés de la LHRH, soit un décapeptide d formule :

$$\underset{3}{Trp} - \underset{4}{Ser} - \underset{5}{Tyr} - \underset{6}{Gly} - \underset{7}{Leu} - \underset{8}{Arg} - \underset{9}{Pro} - \underset{10}{Gly} - NH_2$$

qui possède une grande activité immunogène sans présenter l'activité hormonale de la LHRH naturelle.

L'invention a donc pour objet ce nouveau peptide (3-10) et les conjugués l'incorporant couplé à une protéine porteuse immunogène parmi celles citées plus haut, l'ovalbumine et l'alpha-globuline équine, notamment fractions IV-1 et/ou IV-4, étant préférées.

Dans l'invention, le carbodiimide est préféré au glutaraldéhyde et aux agents hétérobifonctionnels comme agent de conjugaison du peptide LHRH (3-10) notamment sur l'alpha-globuline équine ou l'ovalbumine.

Dans la préparation préférée de conjugué, la LHRH (310) et la protéine porteuse, ovalbumine ou alpha-globuline, sont mises en solution à raison de 2 à 40 mg par ml chacune dans le tampon NaCl 0,1M-acide(N-morpholino)-2 éthane sulfonique 0,1M. Ensuite, 0,5 à 2 volumes de solution de N-éthyl-N'(diméthylamino-3 propyl)carbodiimide à 2,5 % dans le même tampon sont additionnés. Le pH est ajusté par addition de soude 1N. Après agitation, le mélange est laissé une nuit, puis est purifié par chromatographie de perméation sur gel, qui élimine la LHRH (3-10) non couplée, le carbodiimide résiduel et ses produits d'hydrolyse.

L'invention a aussi pour objet les nouveaux vaccins anti-LHRH utilisant de tels conjugués comme principe actif, utilisables pour le procédé selon l'invention.

L'invention a également pour objet l'immunisation passive anti-LHRH (3-10) conformément au procédé décrit plus haut.

Elle a également trait aux ensembles regroupant dans un même emballage un nombre égal de doses de vaccin à administrer avant l'abattage et de vaccin à administrer en première injection. De préférence ces vaccins sont conditionnés sous volume réduit et concentration augmentée pour l'administration par jet transcutané, par exemple selon la demande de brevet français précitée.

L'invention va être maintenant décrite plus en détail à l'aide d'une part d'essais comparant plusieurs produits et procédés de vaccination selon l'invention et d'autre part d'essais ayant montré la prédominance de la réponse immunitaire des porcs mâles à la fraction carboxyterminale du peptide LHRH et de l'essai de vaccination anti-LHRH effectué sur les porcs mâles selon l'invention.

I - UTILISATION DE LA LHRH TOTALE.

A. Plus grande activité immunogène du conjugué maintenant intacte la fraction carboxyterminale la plus étendue du peptide LHRH et choix du conjugué à base de LHRH de formule naturelle de préférence à celui obtenu à l'aide de l'agoniste (D-Lys^6)-LHRH.

A1. - Immunisation anti-LHRH du porc mâle entier et du rat mâle OFA.

La comparaison d'activité de deux vaccins anti-LHRH constitués de conjugués entre la LHRH de forme naturelle (B1 et B2) ou la (D-Lys^6) -LHRH (A1 et A2) et l'albumine humaine, conjugués obtenus par le carbodiimide en phase aqueuse et le SPDP respectivement, mis dans une émulsion huile-dans-l'eau et administrés par voie intramusculaire chez le porc et par voie sous-cutanée chez le rat, conduit aux conclusions suivantes :

- Activité plus grande du vaccin à base de LHRH de forme naturelle : masse du peptide LHRH conjugué inférieure à celle du peptide (D-Lys^6)-LHRH conjugué pour un recrutement d'un plus grand nombre d'animaux présentant une réponse immunitaire (tableaux 1 et 3).
- Effet de dose qui se traduit par un recrutement d'un nombre plus élevé d'animaux présentant une réponse immunitaire par un même conjugué (tableau 3).

A1.1 - Préparation des conjugués (D-Lys^6)-LHRH-albumine par le SPDP.

La préparation des conjugués (D-Lys^6)-LHRH-albumine est réalisée en 3 étapes : préparation de la (N-(pyridyl-2)-dithio-3 propanoyl-D-Lys^6)-LHRH, préparation de la N-(mercapto-3 propanoyl) albumine, puis couplage.

La (N$^{\varepsilon}$ -(pyridyl-2)dithio-3 propanoyl-D-Lys^6)-LHRH est préparée en faisant réagir un excès de SPDP sur la LHRH, en solution aqueuse (6 moles d SPDP par mole de LHRH), puis, après une nuit à 4°C, en centrifugeant le produit obtenu. Celui-ci est dissous dans l'urée 8M et les groupements (pyridyl-2,)dithio présents sont dosés.

La N-(mercapto-3 propanoyl) albumine est obtenue par action de 0,2 mmole de SPDP sur 1 g d'albumine humaine dissoute dans 100 ml de tampon phosphate 0,1M, puis, après une nuit de contact à 4°C et acidification à pH 6, par réduction par le dithiothréitol. Elle est nsuite purifiée par chromatographi de filtration sur gel. Le dosage des thiols et des protéines fournit le niveau de substitution moyen.

Le couplag est effectué en prenant un groupem nt (pyridyl-2)dithi pour 1,25 group ment thiol. Le pH est amené à 7-7,5, puis, un heure après, le rendement est déterminé par mesur de la pyridine thione-2 libé-

rée.

Le niveau de substitution moyen s'en déduit. Finalement, le conjugué est purifié par chromatographie t est concentré par ultrafiltration.

A1.2 - Préparation du conjugué LHRH-albumine par le carbodiimide.

A 300 mg de LHRH et 300 mg d'albumine humain dissous dans 30 ml d NaCl 0,9 % sont additionnés 1000 mg de chlorhydrate de N-éthyl-N'-(diméthylamine-3 propyl) carbodiimide dissous extemporanément dans 40 ml de NaCl 0,9%. Après agitation, le mélange est laissé une nuit à température ambiante à l'abri de la lumière. Ensuite, il est chromatographié sur un gel de Séphadex G-50 ; les fractions correspondant au conjugué sont recueillies, éventuellement concentrées et congelées.

A partir des fractions contenant la LHRH non couplée est déterminée la quantité de LHRH non couplée et donc le niveau de conjugaison moyen. Celui-ci est reproductible et varie de 8 à 10 mg de LHRH couplée pour 100 mg d'albumine.

A partir des spectres UV du conjugué avant et après chromatographie, sont déduits les rendements de la chromatographie en conjugué et donc la quantité (ou la concentration) de LHRH conjuguée.

A1.3 - Techniques de dosage

Le titre en anticorps est déterminé selon la technique décrite par JEFFCOATE et al., Acta. Endocr., Copenh., 1974, 75 : 625-635.

La testostérone est dosée directement sur plasma par une technique RIA utilisant le radioligand Testostérone C19-carboxyméthyl éther (^{125}I)histamine.

La liaison au peptide marqué est déterminée après marquage à l'iode 125 des différents peptides selon COPPOLAND et al., Endocr., 1979, 104 : 1504-1506 et titrage selon la technique décrite par JEFFCOATE et al., Acta Endocr., Copenh., 1974, 75, 625-635.

A.1.4 - Illustrations

– Essais sur rats

. Tableau n° 1 : Réponse anticorps anti-LHRH mesurée par le taux de fixation de LHRH marquée à l'iode^{125}

. Tableau n° 2 : Effet de l'immunisation anti-LHRH sur la concentration de testostérone plasmatique.

. Posologie

Vaccins

A1 : 50 µg de (D-Lys^{6})-LHRH conjuguée

B1 : 12 µg de LHRH conjuguée.

– Essais sur porcs mâles entiers.

. Tableau n° 3 : Réponse anticorps anti-LHRH mesurée par le taux de fixation de LHRH marquée à l'iode^{125}

. Posologie

Vaccins

A1 : 0,5 mg de (D-Lys^{6})-LHRH conjuguée

A2 : 6 mg de (D-Lys^{6}-)LHRH conjuguée

B1 : 0,15 mg de LHRH conjuguée

B2 : 1,20 mg de LHRH conjuguée.

Tableau 1

Réponse anticorps anti-LHRH mesurée par le taux de fixation de LHRH marquée à l'iode^{125}

RECHERCHE DES ANTICORPS (% Bo/T) DANS LE SERUM (1/100)
CHEZ LE RAT

INJECTIONS	TEMPS (SEM)	GROUPE A1 50µg D1ys6-LHRH/HSA/AE1	GROUPE B1 12 µg LHRH/ EDC/HSA/AE1	TITRE B/T=50%
SC	0	-	-	-
		-	-	-
		-	-	-
		-	-	-
SC	4	0.0	15.4	<100
		7.8	7.6	<100
		0.0	35.3	<100
		0.0	51.1	100
	5	4.3	91.1	1600
		14.1	85.3	980
		5.9	70.8	270
		15.8	94.9	2100
	6	0.0	64.3	120
		11.3	87.9	220
		11.7	95.1	2400
		0.0	68.3	280
	7	14.5	72.5	400
		0.0	92.4	2100
		0.0	64.2	200
		11.5	67.2	240
	8	19.6	70.4	310
		0.0	93.8	3200
		9.2	68.7	2200
		0.0	38.8	310

Tableau 2

Effet de l'immunisation anti-LHRH sur la concentration testostérone plasmatique

DOSAGE DE LA TESTOSTERONE PLASMATIQUE (NG/ML)
CHEZ LE RAT

INJECTIONS	TEMPS (SEM)	TEMOINS	GROUPE A1 50 µg Dlysé-LHRH/PDP/HSA	GROUPE B1 12 µg LHRH/EDC /HSA
SC	0	0.40	-	-
		0.26	-	-
		0.47	-	-
		0.00	-	-
SC	4	2.25	4.16	2.51
		1.05	5.83	2.38
		2.34	4.43	3.63
		-	4.17	0.58
	5	3.80	2.99	0.00
		1.76	2.33	0.00
		5.05	2.85	0.00
		6.67	1.54	0.00
	6	2.01	3.26	0.00
		4.47	0.09	0.00
		4.69	1.10	0.00
		1.96	-	0.00
	7	2.28	1.32	0.00
		1.92	0.89	0.00
		1.89	1.59	0.00
		1.65	2.17	0.00
	8	0.97	1.75	0.00
		1.91	1.48	0.00
		2.71	1.91	0.00
		1.22	2.33	0.00

TABLEAU 3

REPONSE ANTICORPS ANTI-LHRH MESUREE PAR LE TAUX DE FIXATION DE LHRH MARQUEE A L'IODE 125

RECHERCHE DES ANTICORPS ANTI-LHRH DANS LE SERUM (DIL. 1/50)

TEMPS (SEM.)	D LYS6-LHRH-POP-HSA/AE1 0.5 µg (A1)					D LYS6-LHRH-POP-HSA/AE1 6 µg (A2)					TITRE (B/T=50%)
	No porcs 211	219	231	243	257	No porcs 205	221	227	245	251	A1 No porcs 221
T0 1ere inj.											
T3	0	0	0	0	0	0	71.8	0	0	0	150
T4	0	0	0	0	0	0	67.3	0	0	0	100
T5 2eme inj.	0	0	0	0	0	0	53.4	0	0	0	50
T6	0	0	0	0	0	14.2	58.5	42.1	6.6	0	55
T7	0	0	0	0	0	7.5	48.5	36.7	5	0	<50
T8	0	0	0	0	0	7.7	44.1	28.8	5	0	<50
T9	0	0	0	0	0	5	37.5	22.6	5	0	<50

TEMPS (SEM.)	LHRH-(CARBO)-HSA/AE1 0,150 mg (B1)					LHRH-(CARBO)-HSA/AE1 1,2mg (B2)					B1	B2	
	No porcs 213	225	247	255	261	No porcs 209	215	235	241	259	No porcs 247	No porcs 235	259
T0 1ere inj.													
T3	0	0	0	0	0	0	0	0	0	0			
T4	0	0	0	0	0	0	0	0	0	0			
T5 2eme inj.	0	0	0	0	0	0	0	0	0	0			
T6	0	0	81.2	5	25.1	48.9	56.2	78.2	31.4	90.1	200	130	990
T7	0	0	81.7	5	49.1	64.7	49.9	86.3	52.3	92.9	170	220	880
T8	0	0	80.5	5	36.5	54.1	48.9	80.7	59.3	90.5	150	140	640
T9	0	0	74.4	5	34.5	54.4	40.1	68.5	51.6	89.1	220	220	490

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A2 - Essai comparatif de deux vaccins anti-LHRH composés respectivement d'un conjugué LHRH-α-globulin par l carbodiimide et d'un conjugué (D-Lys^6)-LHRH-α-globuline par le SPDP mis en émulsion huile-dans-l' au et administrés par voie intramusculaire (IM) ou transcutané (ID) chez le porc.

A2.1 - Préparation de conjugué (D-Lys^6)-LHRH- α - globuline par le SPDP

La méthode décrite dans l'exemple A1 est employée exactement de la même façon, mais en remplaçant l'albumine à 10 mg/ml par l'α-globuline à 6 mg/ml.

Le rendement global en (D-Lys^6)-LHRH couplée est de l'ordre de 45 à 50 %.

Par ailleurs il est possible de modifier à volonté le degré de substitution de l'α -globuline, donc le niveau de conjugaison, en jouant sur les concentrations en SPDP et/ou en α -globuline lors de la préparation de la MP- α - globuline.

A2.2 - Préparation de conjugué LHRH- α -globuline humaine, par le carbodiimide (EDC).

La méthode décrite dans l'exemple A1 est employée exactement de la même façon mais en remplaçant l'albumine humaine par l'α -globuline humaine. Le niveau de conjugaison est de 24 à 28 mg de LHRH fixée pour 100 mg d'α -globuline humaine.

A2.3 - L'efficacité du vaccin à base du conjugué LHRH- α -globuline par le carbodiimide est supérieure au second. L'efficacité est exprimée par le nombre d'animaux présentant une disparition totale de la testostérone plasmatique (tableau 4).

Tableau 4

	LHRH-α-glob.EDC (1,2 mg) IM + ID	[D-Lys^6]-LHRH-α-glob.SPDP (6 mg) IM + ID
Suppression de testostérone	5/10	2/10

A.3 - Prédominance de la réponse immunitaire des porcs mâles à la fraction carboxyterminale du peptide LHRH conjugué par le carbodiimide ou de son agoniste (D-Lys^6)-LHRH conjugué par le SPDP sur l'α -globuline humaine.

Elle est déterminée par la comparaison des pourcentages de fixation des sérums anti-LHRH et anti-(D-Lys^6)-LHRH par deux fragments marqués de LHRH, respectivement LHRH (3-10) délété de sa fraction amino-terminale et LHRH (1-10) sous forme d'acide libre et de ce fait délété de la fraction amide de sa fraction carboxyterminale naturelle. Ces deux fractions reconnaissent respectivement plus particulièrement les anti-corps dirigés contre les fractions carboxyterminale d'une part, et aminoterminale d'autre part.

La prédominance de la réponse à la fraction carboxyterminale du peptide se traduit par un nombre d'animaux présentant des anticorps ne fixant que le peptide LHRH (3-10) à l'exclusion de la fixation de LHRH acide libre (10/58 pour le sérum anti-LHRH et 3/10 pour le sérum anti-(D-Lys^6)-LHRH).

Aucun sérum n'a montré de fixation à 100 % de la fraction LHRH acide libre, laquelle traduirait une reconnaissance exclusive de la fraction aminoterminale.

Les réponses mixtes, les plus fréquentes, montrent une meilleure reconnaissance de la fraction aminoterminale par les sérums anti-(D-Lys^6)-LHRH que celle des sérums anti-LHRH. Chez ces derniers, seuls 3 sérums sur 58 ont une reconnaissance supéri ure à 40 % de la fraction aminoterminale, contre 4 sur 10 pour le sérum anti-(D-Lys^6)- LHRH.

B - Plus grande activité immunogène du conjugué LHRH- α -globuline réalisé par le cabodiimide, comparé à celle qui est obtenue par l conjugué préparé avec le glutaraldéhyde.

B.1 - Préparati n du conjugué LHRH- α -globuline par le glutaraldéhyde.

A 10 mg de LHRH et 50 mg d'α -globuline humaine (Serva) dissous dans 5 ml de tampon phosphate 0,1M pH 7,5 sont ajoutés, goutte à goutte et sur une durée de 30 mn, 2,5 ml de solution de glutaraldéhyde à 10 mg/ml, n agitant doucement après chaque addition. Après avoir laissé le mélange 2,5 h à températur ambiante, la réaction est arrêté par addition de 25 mg de bisulfit d sodium dissous dans 0,5 ml d'eau. Le conjugué est

dialysé à 4°C contre le tampon NaCl 150mM-phosphate 10mM pH 7,5, puis est concentré par ultrafiltrati n.

B.2 - Essai comparatif sur le porc de vaccins anti-LHRH formulés à l'aide de quantités identiques de LHRH conjuguée. L'efficacité est xprimée par le nombre d'animaux présentant un disparition totale de la testostérone plasmatique (tableau 7).

Tableau 7

	LHRH-α-glo. par carbodiimide administration IM ou ID	LHRH-α-glo. par glutaraldéhyde administration IM ou ID
Suppression de testostérone plasmatique	5/10	0/10

C - Plus grande activité immunogène du conjugué utilisant l'α -globuline humaine comparée à celle qui est obtenue par le conjugué utilisant de la sérumalbumine humaine.

L'efficacité est exprimée par le nombre d'animaux présentant une disparition totale de la testostérone plasmatique (tableau 8).

Tableau 8

Essais sur porcs - injection intramusculaire

	LHRH-HSA par carbodiimide	LHRH-α-glo. par carbodiimide
Suppression de testostérone plasmatique	0/5	3/5

D - Activité immunogène du conjugué utilisant l'α-globuline équine, fraction IV-1, équivalente à celle qui est obtenue par le conjugué utilisant l'α -globuline humaine.

D.1 - Préparation de conjugué LHRH- α -globuline équine par le carbodiimide.

La méthode décrite dans l'exemple A1 est employée exactement de la même façon, mais en remplaçant l'albumine humaine par l'α -globuline équine (fraction IV-1).

D.2 - Administration chez le rat par voie sous-cutanée à 2 reprises à 4 semaines d'intervalle d'un vaccin à la dose de 12 µg de LHRH conjuguée à l'α -globuline humaine ou équine.

Tableau 9

Essais sur rats

	LHRH-α-globuline humaine fraction IV-1 par carbodiimide	LHRH-α-globuline équine fraction IV-1 par carbodiimide
Suppression de la testostérone plasmatique	12/12	12/12

E - Plus grande activité adjuvant de l'émulsion eau-dans-l'huile de l'inv ntion sur d'autres émulsions

(tableau 10).

Essais sur le porc en utilisant le même conjugué composé de la LHRH et de l'α-globuline humaine par le carbodiimide et administré à la même dose sous un même volume par la voie transcutanée en 5 points.

Les émulsions examinées sont : une émulsion fluide huile-dans-l'eau (B), l'émulsion de l'invention (formule C du tableau), une émulsion du commerce à diluer avec l'antigène (E), une phase huileuse à émulsionner avec le conjugué (F).

Pour toutes ces formules, la quantité finale d'antigène par dose est la même.

Les émulsions sont réalisées dans les conditions habituelles pour ceux experts en formulation de ce type.

Tableau 10

Emulsions	B	C	E	F
Suppression de la testostérone plasmatique	2/5	4/4	1/5	3/5
Nombre d'animaux présentant une concentration de l'androsténone tissulaire au-dessous de 0,5 µg/g	2/5	4/4	3/5	3/5

F. Efficacité de l'immunisation passive anti-LHRH pour l'amélioration des qualités organoleptiques de la viande, mesurée par l'abaissement de l'androsténone tissulaire.

Tableau 11

Teneur en adrosténone du tissu adipeux chez les animaux témoins et chez ceux qui ont été soumis à l'immunoneutralisation anti-LHRH passive par un plasma hyperimmun équin anti-(D-Lys^6)-LHRH administré sous un volume de 300 ml aux jours 16, 13, 9 et 5 avant abattage.

	Témoins	Traités
Nombre d'animaux présentant une concentration d'androsténone inférieure à 0,50 µg/g de tissu adipeux	2/5	5/5

(différence significative au risque $\alpha = 0,2$)

G. - Efficacité et tolérance des formulations renfermant la LHRH conjugué à l'α-globuline par le carbodiimide en émulsion eau-dans-l'huile (1er vaccin) et en gel d'hydroxyde d'aluminium et saponine (2ème vaccin), administrées à la même dose de LHRH conjuguée, respectivement au début de la mise en engraissement et 18 à 21 jours avant l'abattage par voie transcutanée à l'aide d'un injecteur sans aiguille dénommé Pigjet.

Deux essais ont été effectués en deux temps, respectivement les groupes 1, 3 et 5 pour le premier et les groupes 2 et 4 pour le second (tableaux 12 et 13).

G.1 - L'efficacité de l'immunoneutralisation, anti-LHRH est augmentée pour un volum égal de vaccin par la multiplication des points d'administration transcutanée.

Tableau 12

Groupes	1	2	3	4	5
1er vaccin	1 ml (5 points)	1 ml (5 points)	1 ml (5 points)	0,4 ml (10 points)	0,4 ml (2 points)
2e vaccin	1 ml (5 points)	1 ml (5 points)	0,4 ml (2 points)	0,4 ml (10 points)	0,4 ml (2 points)
Suppression ou diminution marquée de la testostérone (nbre d'animaux)	10/12	10/11	9/12	11/11	8/11
Concentration de l'androsténone tissulaire au-dessous de 0,5 µg/g (nbre d'animaux)	11/12	ND	10/23	ND	ND

ND : non déterminé

G.2 - La tolérance aux vaccins utilisés est jugée par l'évolution de la réaction inflammatoire cutanée, notée de 0 à 4 chez un animal en fonction de l'importance des papules apparaissant après l'administration ; une papule apparaît à chaque point d'administration. La sommation des notes dans chacun des groupes est résumée comme suit : note moyenne à l'issue de la première semaine qui suit l'administration (Ad. 1) et note moyenne au moment de l'abattage pour chacun des vaccins (Ab) (tableau 13). La meilleure tolérance est observée avec l'emploi des vaccins dans le groupe 4.

Tableau 13

Tolérance par administration par voie transcutanée observée au cours des 2 essais effectués (essai 1 groupes 1, 3 et 5, essai 2 groupes 2 et 4).

Groupes	1		2		3		4		5	
Vaccins 1 ou 2	1e vac.	2e vac.	1e vac.	2e vac.	1e vac.	2e vac	1e vac.	2e vac.	1e vac.	2e vac.
Nombre de points d'administration	5	5	5	5	5	2	10	10	2	2
Ad 1	42	11	31	11	41	16	33	11	30	10
Ab	2	4	0	0	5	3	0	0	2	0
Nombre d'animaux	12		11		12		11		11	

II - UTILISATION DU PEPTIDE (3-10).

A. Techniques de la mesure de la réponse immunitaire anti-LHRH et de l' fficacité biologique par le dosage d la testostérone plasmatique et de l'androstérone tissulaire.

La réponse immunitaire anti-LHRH est mesurée par le titre n anticorps qui est dét rminé selon la technique décrite par JEFFCOATE et al., Acto. Endocr. (Copenh.), 1974, 75, 625-635.

La liaison aux peptides marqués est déterminée après marquage à l'iode 125 des différents peptides selon COPPOLAND et al., Endocrinology., 1979, 104, 1504-1506. Le titrage des sérums vis-à-vis de ces peptides est effectué selon la technique de JEFFCOATE et al. citée ci-dessus.

L'efficacité biologique est mesurée par l'abaissement ou la disparition de la testostèrone plasmatique et de l'androsténone tissulaire. Le dosage de la testostérone plasmatique est effectué directement sur le plasma par une technique RIA utilisant le radioligand testostérone C19-carboxyméthyl éther (^{125}I) histamine (FURUYAMA S. et al., Steroids, 1972, 16, 415). Le dosage de l'androsténone tissulaire est effectué sur un échantillon de tissu adipeux par une technique RIA utilisant le radioligand 5α-^{3}H-androsténone, décrite par CLAUS, C.R. Acad. Sci., Paris, 1974, 278, 299-302.

B. Prédominance de la réponse immunitaire des porcs mâles à la fraction carboxyterminale du peptide LHRH conjugué par le carbodiimide ou de son agoniste [D-Lys^{6}]-LHRH conjugué par le SPDP sur l'α-globuline humaine.

B1. Préparation du conjugué LHRH-a-globuline humaine par le carbodiimide.

Le conjugué est de préférence obtenu par addition à un volume du mélange α -globuline et LHRH, en solution à 2 à 20 mg/ml dans NaCl 0,9%, de 0,5 à 2 volumes de solution de chlorhydrate de N-éthyl-N'-(diméthylamino-3 propyl)carbodiimide (EDC) en solution à 2,5% dans NaCl 0,9%. Après agitation, le mélange est laissé une nuit, puis purifié par chromatographie de perméation sur gel.

B2. Préparation des conjugués ([D.Lys^{6}]-LHRH)-α-globuline humaine par le SPDP.

La préparation des conjugués ([D.Lys^{6}]-LHRH)-α-globuline humaine est réalisée en 3 étapes : préparation de la [N-(pyridyl-2)-dithio-3 propanoyl-D-Lys^{6}]-LHRH, préparation de la N-(mercapto-3 propanoyl) α-globuline humaine, puis couplage.

La [N$^{\varepsilon}$-(pyridyl-2)dithio-3 propanoyl-D-Lys^{6}]-LHRH est préparée en faisant réagir un excès de SPDP sur la LHRH, en solution aqueuse (6 moles de SPDP par mole de [D-Lys^{6}]-LHRH, puis, après une nuit à 4°C, en centrifugeant le produit obtenu. Celui-ci est dissous dans l'urée 8M et les groupements (pyridyl-2)dithio présents sont dosés.

La N-(mercapto-3 propanoyl) α-globuline humaine est obtenue par action de 0,2 mmole de SPDP sur 0,6 g d'α-globuline humaine dissoute dans 100 ml de tampon phosphate 0,1M, puis, après une nuit de contact à 4°C et acidification à pH 6, par réduction par le dithiothréitol. Elle est ensuite purifiée par chromatographie de filtration sur gel. Le dosage des thiols et des protéines fournit le niveau de substitution moyen.

Le couplage est effectué en prenant un groupement (pyridyl-2)dithio pour 1,25 groupement thiol. Le pH est amené à 7-7,5, puis, une heure après, le rendement est déterminé par mesure de la pyridine thione-2 libérée.

Le niveau de substitution moyen s'en déduit. Finalement, le conjugué est purifié par chromatographie et est concentré par ultrafiltration. Le rendement global en [D-Lys^{6}]-LHRH couplée est de l'ordre de 45 à 50%.

B3. La prédominance de la réponse immunitaire des porcs mâles à la fraction carboxyterminale du peptide LHRH conjugué dans les conditions décrites en A1 et A2 est déterminée par la comparaison de la fixation par les sérums anti-LHRH et anti-[D-Lys^{6}]-LHRH, de deux fragments marqués de LHRH, respectivement LHRH (3-10), (LHRH délétée de sa fraction aminoterminale) et LHRH (1-10) sous forme d'acide libre, (LHRH délétée de la fraction amide de sa fraction carboxyterminale). Ces deux fragments reconnaissent plus particulièrement les anticorps dirigés respectivement contre les fractions carboxyterminale d'une part, et aminoterminale d'autre part.

La réponse à la fraction carboxyterminale du peptide est générale sur tous les animaux immunisés par l'un ou l'autre des conjugués (68/68). Les sérums de 3 sur 10 des 10 animaux immunisés par la [D-Lys^{6}]-LHRH conjuguée et de 10 sur 68 des animaux immunisés par la LHRH conjuguée ont montré exclusivem nt une fixation de la fraction carboxyterminal . Les autres animaux présentent une réponse mixte dirigée préférentiellement contre la fraction carboxyt rminale.

La réponse à la fraction aminoterminal n'est pas générale (55/68). Aucun sérum n'a montré d fixation exclusiv à la fraction LHRH acide libre.

C. Essais d'immunoneutralisation activ anti-LHRH à l'aide des conjugués LHRH (3-10)-α-globuline équine IV-4 et LHRH (3-10)-ovalbumine réalisés par l carbodiimide.

C1. Préparation du conjugué LHRH (3-10)-α-globulin équine IV-4 par l carbodiimide.

Quatre-vingt-cinq mg de LHRH (3-10) t 170 mg d'α-globuline équine IV-4 sont diss us dans 12,8 ml d

tampon NaCl 0,1M - acid (N-m rpholino)-2 éthanesulfonique 0,1M. Puis 212 mg de chlorhydrat de N-éthyl-N'-(diméthylamino-3 propyl)carbodiimide dissous dans 17 ml de solution précéd nt sont ajoutés. L pH est immédiatem nt ajusté à 6,0 par addition de 1,3 ml de soude 1N.

Après agitation, l mélange est laissé 16h à température ambiante, puis le conjugué est purifié par chromatographie de perméation sur gel pour séparer le conjugué de la LHRH non conjuguée. La mesure de la quantité de cette dernière permet d'avoir par différence la quantité de LHRH couplée. Il est possible de déterminer le rendement de couplage.

C2. Préparation du conjugué LHRH (3-10)-ovalbumine par le carbodiimide.

Soixante mg de LHRH (3-10) et 120 mg d'ovalbumine sont dissous dans 9 ml de tampon NaCl 0,1M - acide (N-morpholino)-2 éthanesulfonique 0,1M. Puis 150 mg de chlorhydrate du N-éthyl-N'-(diméthylamino-3 propyl)carbodiimide dissous dans 12 ml du même tampon sont ajoutés. Le pH est ajusté à 7,0 par addition de soude 1N (1,9 ml environ). Le mélange est laissé une nuit à température ambiante, il est ensuite clarifié par centrifugation. Le surnageant est chromatographié sur gel de Séphadex pour séparer le conjugué de la LHRH n'ayant pas réagi et des produits provenant du carbodiimide initial. Par mesure de la quantité de LHRH (3-10) non fixée, il est possible de déterminer le rendement de couplage de la LHRH (3-10).

C3. Réponse immunitaire, efficacité biologique et tolérance au vaccin anti-LHRH formulé à partir du conjugué obtenu entre les fragments LHRH (3-10) et l'α-globuline équine IV-4 par le carbodiimide.

Les formulations constituées de la LHRH (3-10) conjuguée mises en émulsion eau-dans-l'huile (1er vaccin) et en gel d'hydroxyde d'aluminium et saponine (2e vaccin) ont été administrées à 6 porcs mâles sous un volume de 0,4 ml par dose, respectivement au début de la mise en engraissement et 17 jours avant l'abattage par voie transcutanée à l'aide d'un injecteur sans aiguille dénommé Pigjet délivrant le volume de la dose en 2 applications de 0,2 ml réparties en 5 points à chacune d'elles.

La réponse immunitaire fut maximale 10 jours après l'administration du 2e vaccin. Les titres individuels en anticorps (inverse de la dilution par laquelle l'iode 125 est fixé à 50%) furent respectivement :

Jour 10	280	660	2 700	3 200	4 600	13 000
Jour 16	290	400	2 000	2 400	3 100	8 600

L'efficacité biologique de cette réponse immunitaire s'est traduite par la disparition de la testostérone plasmatique dès le 10e jour après l'administration du 2e vaccin chez tous les 6 animaux. La disparition de la testostérone s'accompagne, dans les mêmes conditions, de la disparition de l'androsténone tissulaire.

La tolérance au vaccin est jugée par l'évolution de la réaction inflammatoire cutanée, notée en fonction de l'importance des papules apparaissant à chaque point de délivrance du vaccin après administration. Cette inflammation locale a totalement disparu dès le jour 10 après l'administration du 2e vaccin.

Revendications

1. Procédé pour améliorer les qualités organoleptiques, en particulier l'odeur, la sapidité et la tendreté, de la viande des animaux domestiques mâles non castrés, dans lequel, peu avant l'abattage de l'animal concerné, on supprime sensiblement l'action des stéroïdes androgènes et non androgènes, par immunoneutralisation active ou passive anti-LHRH, tout en maintenant pratiquement jusqu'à l'abattage les avantages liés au caractère mâle de l'animal.

2. Procédé selon la revendication 1, caractérisé en ce qu'on administre en premier lieu un vaccin anti-LHRH, puis, peu avant l'abattage de l'animal, on administre à nouveau un vaccin anti-LHRH.

3. Procédé selon la revendication 2, caractérisé en ce que l'on administre en premier lieu un vaccin conçu pour induire une première réponse immunitaire de faible intensité, sans effet notable, ou même mesurable, sur la sécrétion des stéroïdes gonadiques et en c que, avant l'abattag , on administre un vaccin formulé pour produire la suppression ou l'abaissement significatif de la sécrétion des stéroïdes sans réaction locale ou générale adv rse, susceptible de nuire à l'apparence ou à la qualité d la viand .

4. Procédé selon l'une quelconque des revendications 2 t 3, caractérisé en ce que le vaccin anti-LHRH administré en premier lieu l'est avant la phase d'engraissem nt de l'animal.

5. Procédé selon l'une qu lconque des revendicati ns 2 à 4, caractérisé en ce que le vaccin anti-LHRH admi-

nistré en premier lieu st un vaccin en émulsion.

6. Procédé selon l'un quelconqu des rev ndications 2 à 5, caractérisé en ce que, pour l porc, on administre, avant l'abattage, le vaccin anti-LHRH av c un adjuvant de type aqueux.

7. Procédé selon la revendication 6, caractérisé en ce que, comme adjuvant de type aqueux, on utilise du gel d'hydroxyde d'aluminium et/ou de la saponine.

8. Procédé selon la revendication 6 ou 7, caractérisé en ce qu'on administre le vaccin en adjuvant aqueux de 15 à 21 jours avant l'abattage.

9. Procédé selon l'une quelconque des revendications 2 à 5, caractérisé en ce que, pour les bovins et les ovins, on administre, avant l'abattage, un vaccin anti-LHRH avec un adjuvant en émulsion.

10. Procédé selon la revendication 9, caractérisé en ce qu'on administre le vaccin en émulsion de un à deux mois avant l'abattage.

11. Procédé selon la revendication 9 ou 10, caractérisé en ce qu'on administre le vaccin en émulsion de quatre semaines à plusieurs mois après l'administration faite en premier lieu.

12. Procédé selon l'une quelconque des revendications 4 et 9 à 11, caractérisé en ce que le vaccin en émulsion est un vaccin en émulsion eau-dans-l'huile.

13. Procédé selon la revendication 12, caractérisé en ce que l'émulsion eau-dans-l'huile est faite d'un mélange d'huiles minérales hautement purifiées et de tensioactifs. non ioniques.

14. Procédé selon l'une quelconque des revendications 2 à 13, caractérisé en ce que l'on administre un conjugué immunogène anti-LHRH comprenant :
   - la LHRH totale ou modifiée,
   - un fragment peptidique de LHRH modifié ou non, ou
   - un agoniste de la LHRH, couplé à une protéine porteuse immunogène choisie parmi :
   - sérum albumine bovine ou humaine,
   - thyroglobuline,
   - ovalbumine, les anatoxines, notamment l'anatoxine tétanique,
   - globulines humaines ou équines.

15. Procédé selon la revendication 14, caractérisé en ce que le conjugué comprend la LHRH couplée à l'alpha-globuline équine, notamment fractions IV-1 et/ou IV-4.

16. Procédé selon la revendication 14, caractérisé en ce que le conjugué comprend la LHRH (3-10) couplée à l'alpha-globuline équine, notamment les fractions IV-1 et/ou IV-4, ou à l'ovalbumine.

17. Procédé selon la revendication 15 ou 16, caractérisé en ce que la LHRH/LHRH (3-10) et la protéine porteuse immunogène sont couplées par un carbodiimide.

18. Procédé selon la revendication 1, caractérisé en ce qu'on administre à l'animal, quelques jours avant l'abattage, du sérum ou du plasma hyperimmun anti-LHRH.

19. Procédé selon la revendication 1, caractérisé en ce qu'on administre à l'animal, quelques jours avant l'abattage, des anticorps monoclonaux anti-LHRH.

20. Procédé selon la revendication 18 ou 19, caractérisé en ce que l'administration est faite de cinq à quinze jours avant l'abattage, par voie sous-cutanée ou intramusculaire.

21. Procédé selon l'une quelconque des revendications 2 à 17, caractérisé n ce qu'on administre par voie transcutanée, d préférence en plusieurs points, à l'aide d'un appar il d'injection sans aiguille par jet sous pression.

22. Peptide d formule :
Trp - Ser - Tyr - Gly - Leu - Arg - Pro - Gly - NH_2.

23. Conjugué comprenant l peptide selon la revendication 22, couplé à un protéine porteuse immunogène.

24. Conjugué selon la revendication 23, caractérisé en ce qu la protéine porteuse immunogène est choisie parmi l'ovalbumine, les globulines équines t humaines, la thyroglobuline, les anatoxines, notamment l'anatoxin tétanique, et les sérumalbumines humaine et bovine.

25. Conjugué selon la revendication 24, caractérisé en ce que la protéine porteuse immunogène du groupe des globulines est l'alpha-globuline équine, notamment fraction IV-1 et/ou IV-4.

26. Conjugué selon l'une quelconque des revendications 23 à 25, caractérisé en ce que le peptide et la protéine porteuse immunogène sont couplés par un carbodiimide.

27. Vaccin anti-LHRH conçu pour induire une réponse immunitaire de faible intensité, sans effet notable, ou même mesurable, sur la sécrétion des stéroïdes gonadiques et comprenant comme principe actif un conjugué alpha-globuline-LHRH ou un conjugué selon l'une quelconque des revendications 23 à 26.

28. Vaccin anti-LHRH conçu pour produire la suppression ou l'abaissement significatif de la sécrétion des stéroïdes sans réaction locale ou générale adverse, susceptible de nuire à l'apparence ou à la qualité de la viande, et comprenant comme principe actif un conjugué alpha-globuline-LHRH ou un conjugué selon l'une quelconque des revendications 23 à 26.

29. Vaccin anti-LHRH selon la revendication 27 ou 28, caractérisé en ce qu'il est en émulsion eau-dans-l'huile.

30. Vaccin anti-LHRH selon la revendication 29, caractérisé en ce que l'émulsion comprend un mélange d'huiles minérales hautement purifiées et de tensioactifs. non ioniques.

31. Vaccin anti-LHRH selon la revendication 27 ou 28, caractérisé en ce qu'il est en adjuvant aqueux.

32. Vaccin anti-LHRH selon la revendication 31, caractérisé en ce qu'il comprend un gel d'hydroxyde d'aluminium et/ou de la saponine.

33. Ensemble de vaccination anti-LHRH comprenant dans un même emballage un nombre égal de doses d'un vaccin à administrer en première injection et d'un vaccin à administrer avant l'abattage, selon l'une quelconque des revendications 27 à 32.

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(57) Abstract

The present invention relates generally to an immunogenic LHRH composition and more particularly to an immunogenic LHRH composition comprising an LHRH C-terminal fragment of at least five amino acids. The present invention is useful, *inter alia*, as a prophylactic and/or therapeutic agent for the modification of fertility and behaviour patterns of animals, the achievement of livestock production gains such as increasing growth, decreasing feed conversion ratios or the control of unwanted organoleptic characteristics or the treatment of disorders of the reproductive organs.

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IMMUNOGENIC LHRH COMPOSITIONS AND METHODS RELATING THERETO

The present invention relates generally to an immunogenic LHRH composition and more particularly to an immunogenic LHRH composition comprising a LHRH C-terminal fragment of at least five amino acids. The present invention is useful, *inter alia*, as a prophylactic and/or therapeutic agent for the modification of fertility and behaviour patterns of animals, the achievement of livestock production gains such as increasing growth, decreasing feed conversion ratios or the control of unwanted organoleptic characteristics or the treatment of disorders of the reproductive organs.

Bibliographic details of the publications referred to by author in this specification are collated at the end of the description. Sequence Identity Numbers (SEQ ID NOs.) for the amino acid sequences referred to in the specification are defined following the bibliography.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Vaccination against the hypothalamic hormone luteinising hormone releasing hormone (referred to herein as "LHRH", also known as GnRH) has been demonstrated as an immunological method of controlling reproduction since the early 1970's (Fraser 1975, Jeffcoate et al 1982). Eliciting an immune response to LHRH prevents the release from the anterior pituitary of the hormones LH and FSH, which are required for the development and maintenance of the gonads - the testes in the male and ovaries in the female. Thus reduction of LH and FSH levels leads to loss of reproductive function.

De-sexing (or neutering) operations are the most widely practised surgical procedures in veterinary medicine and livestock animal management. A significant proportion of both sexes

of domestic livestock and companion animals are routinely surgically de-sexed to prevent a variety of undesirable characteristics which accompany sexual maturity. The traits include fighting, wandering, sexual behaviour, loss of condition, tumours of reproductive organs and pregnancy.

The control of mating behaviour by vaccination with LHRH-conjugate vaccines in companion animals such as dogs, cats and horses, and in livestock specifically in male pigs and male and female cattle, has been identified by the inventor as a goal as significant as the control of fertility.

Similarly, the control and treatment of disorders of the gonads and other reproductive organs, of both humans and animals, such as testicular cancer, breast cancer, prostate cancer, ovarian cancer, prostate enlargement or endometriosis is of significance.

The first published report of vaccination with an LHRH conjugate vaccine in rabbits showed that a dramatic effect was achieved in the development of the testes. Early reports of the application of an LHRH vaccine in pigs (Falvo et al, 1986, Caraty and Bonneau 1986), showed that effective formulations based on 1-10 LHRH conjugated to human serum globulin or bovine serum albumin could control testes development and boar taint. Awonyi et al. (1988) showed that the effect of vaccination of pigs against LHRH affected primarily testis development. All these trials were done on small numbers of animals, with no reports of efficacy.

The problems of variability of LHRH-conjugate vaccines in controlling boar taint have been attempted to be overcome by genetically incorporating LHRH amino acid sequences into carrier proteins, including the pilin gene from *E.coli* (Zee et al 1995) and into a truncated leucotoxin gene from *Pasteurella haemolytica* (Potter et al 1997). These fusion proteins are produced as recombinant molecules and not by biochemical coupling. Trials have shown these recombinant proteins to function as immunocastration vaccines. However, they have not resulted in commercially available vaccines and press reports suggest less than desired

efficacy.

In keeping with the less than perfect nature of highly developed and widely applied subunit vaccines for disease prevention, immunocastration vaccines based on specific LHRH- protein conjugates have also been shown to be less than perfect at inducing antibody to LHRH or in reducing hormones or other parameters associated with reproductive functions. There has been a general recognition of a wide variation in the effective induction of antibody to LHRH with a variety of LHRH-conjugate vaccines (Meloen et al 1994).

Vaccination of cattle with a 1-10 LHRH peptide - human serum albumin conjugate in Freunds adjuvant (Robertson et al, 1982), gave good antibody responses to LHRH after 2 vaccinations in only 5 of 10 vaccinated cattle. Even with boost vaccinations, the poor responders did not maintain antibody titres or have suppressed testosterone. A commercially developed vaccine for cattle (Vaxstrate), was only 80% effective (Hoskinson et al 1990).

Experiments in mice (Sad et al 1991) have shown that responses to LHRH-conjugates are genetically based. The vaccine was a 1-10 LHRH peptide, with the substitution of D-lysine instead of L-glycine at the 6 position, conjugated to diphtheria toxoid and adjuvanted with alum. Some strains of mice responded well, while others showed suppression of antibody to LHRH. These results would lead those skilled in the art of vaccine formulation to expect that a significant proportion of an outbred population would fail to respond or respond poorly to an LHRH-conjugate subunit type vaccine.

Vaccination of male pigs has resulted in variable suppression of testis development and suppression of boar taint. Bonneau and coworkers have shown (Bonneau et al 1994) that a 1-10 LHRH -α globulin conjugate given in oil emulsion for primary vaccination and saponin adjuvant for boost vaccination gave an antibody response in only 90% of 20 vaccinated pigs. Testosterone levels were suppressed in only 16/20 vaccinates (75%). Thus the quality as well as the amount of antibody is important in determining the efficacy of an LHRH-conjugate based vaccine. Hagen et al (1988) claimed that vaccination of 6 boars with an LHRH-bovine

serum albumin (BSA) conjugate in Freunds adjuvant could reduce boar taint. However, 2/6 boars had low antibody responses and had normal spermatogenesis and testis function. Skatole levels were not affected by vaccination against LHRH. Accordingly, there is a need to develop an LHRH vaccine which is consistently more highly effective than those utilized to date.

In work leading up to the present invention, the inventor has determined that the efficacy of vaccination against LHRH is significantly improved when LHRH is administered as a conjugate with diphtheria toxoid and an ionic polysaccharide.

Accordingly, one aspect of the present invention relates to a preparation for use in eliciting an effective immune response to LHRH, said preparation comprising a LHRH-diphtheria toxoid conjugate adsorbed to an ionic polysaccharide.

Reference to an "ionic polysaccharide" should be understood as a reference to any positively or negatively charged polysaccharide or derivative or chemical equivalent thereof. Reference to "derivative" and "chemical equivalent" should be understood to have the same meaning as outlined below. Said ionic polysaccharide may be in soluble or insoluble form. Preferably said ionic polysaccharide is an ionic dextran. Even more preferably said ionic dextran is DEAE-dextran, dextran sulphate or QAE-dextran. Most preferably, said ionic dextran is DEAE dextran. Preferably, the dextran component of said ionic dextran exhibits a molecular weight in the range 250,000 to 4,000,000 Da and even more preferably 500,000 to 1,500,000 Da.

According to this most preferred embodiment, the present invention relates to a preparation for use in eliciting an effective immune response to LHRH, said preparation comprising a LHRH-diphtheria toxoid conjugate adsorbed to DEAE-dextran.

Reference to an "effective" immune response should be understood as a reference to an immune response which either directly or indirectly leads to the reduction or complete blocking of reproductive function (i.e. reduces or prevents the functioning of any one or more of the reproductive organ's activities or modulates the hormonal levels of an animal such that any one or more aspects of reproduction or reproductive activity are reduced) in at least 90%, and preferably at least 95%, of animals treated. It should be understood that efficacy is a functional measure and is not defined by reference to anti-LHRH antibody titre alone since the presence of circulating antibody alone is not necessarily indicative of the capacity of said circulating antibody to block reproductive function. The term "reproductive organ" should be understood in its broadest sense to refer to the male and female gonads and accessory sexual organs. "Accessory sexual organs" should also be understood in its broadest sense and includes, for example, the prostate, breasts and the uterus.

Reference hereinafter to "LHRH" should be read as including reference to all forms of LHRH and derivatives thereof.

"Derivatives" include fragments, parts, portions, chemical equivalents, mutants, homologs and analogs from natural, synthetic or recombinant sources, including fusion proteins. For example, said LHRH includes peptides comprising a sequence of amino acids substantially as set forth in SEQ ID NO:1 or SEQ ID NO:2 or SEQ ID NO:3 or SEQ ID NO:4 or having at least 50% similarity thereto. The molecules defined in SEQ ID Nos:1, 2 and 3 are from the human and are conserved across all mammals. SEQ ID NO:4 is a derivative of SEQ ID NO:2 wherein spacers have been introduced at the N-terminus. Chemical equivalents of LHRH can act as a functional analog of LHRH. Chemical equivalents may not necessarily be derived from LHRH but may share certain similarities. Alternatively, chemical equivalents may be specifically designed to mimic certain physiochemical properties of LHRH. Chemical equivalents may be chemically synthesised or may be detected following, for example, natural product screening.

Homologs of LHRH contemplated herein include, but are not limited to, LHRH derived from different phyla including birds, fish, reptiles and invertebrates.

"Derivatives" may also be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acids sequence variants are those in which one or more amino acid or non-natural amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different natural or non-natural residue inserted in its place. Typical substitutions are those made in accordance with Table 1:

TABLE 1

Suitable residues for amino acid substitutions

	Original Residue	Exemplary Substitutions
	Ala	Ser
*	Arg	Lys
	Asn	Gln; His
	Asp	Glu
	Cys	Ser
	Gln	Asn
*	Glu	Ala
*	Gly	Pro
*	His	Asn; Gln
	Ile	Leu; Val
*	Leu	Ile; Val
	Lys	Arg; Gln; Glu
	Met	Leu; Ile
	Phe	Met; Leu; Tyr
*	Ser	Thr
	Thr	Ser
*	Trp	Tyr
*	Tyr	Trp; Phe
	Val	Ile; Leu

Key: Amino acid residues marked with an asterisk indicate residues present in the mammalian LHRH sequence.

Examples of non-natural amino acids include, but are not limited to the D-isomers of said amino acids. "Additions" to amino acid sequences include fusion with other peptides, polypeptides or proteins.

Reference to diphtheria toxoid should be understood as a reference to all forms of diphtheria toxoid and derivatives thereof. The term "derivatives" has the same meaning as hereinbefore defined. Derivatives may include, for example, molecules comprising the diphtheria toxoid T cell epitopes or said T cell epitopes in isolation.

Preferably, said LHRH comprises an LHRH C-terminal fragment of at least five amino acids. Even more preferably, said LHRH comprises the amino acid sequence substantially as set forth in SEQ ID NO:2 and wherein the carboxyl terminus of said amino acid sequence is amidated. Said preferred LHRH is referred to herein as "LHRH 2-10 form".

According to this most preferred embodiment there is provided a preparation for use in eliciting an effective immune response to LHRH said preparation comprising a LHRH 2-10 form-diphtheria toxoid conjugate adsorbed to DEAE dextran.

In another preferred embodiment said LHRH comprises the amino acid sequence substantially as set forth in SEQ ID NO:4. Said preferred LHRH is referred to herein as "modified LHRH 2-10 form".

According to this preferred embodiment there is provided a preparation for use in eliciting an effective immune response to LHRH said preparation comprising a modified LHRH 2-10 diphtheria toxoid conjugate adsorbed to DEAE dextran.

Although not intending to limit the invention to any one method, said peptide may be synthesised by Fmoc chemistry and the resulting peptide coupled to the carrier protein diphtheria toxoid. Said coupling may be performed as described in Ladd *et al* 1990 or in Bonneau *et al* 1994, and the resulting conjugate of peptide and carrier protein (referred to herein as "peptide-conjugate") processed to be free of unbound peptide and other biproducts of conjugation. Such processing may be achieved by conventional dialysis or by ultrafiltration. The resulting peptide-conjugate is adsorbed to the ionic polysaccharide adjuvant.

Without limiting the present invention to any one theory or mode of action, administration of an effective amount of the LHRH preparation of the present invention induces a significantly more effective immune response against LHRH than the LHRH-carrier-adjuvant compositions described in the prior art. Said improved efficacy is observed when the immunogenic LHRH composition specifically comprises the carrier diphtheria-toxoid and an ionic polysaccharide adjuvant.

In another aspect of the present invention there is provided a pharmaceutical composition comprising a LHRH-diphtheria toxoid conjugate adsorbed to an ionic polysaccharide together with one or more pharmaceutically acceptable carriers and/or diluents.

Preferably said ionic polysaccharide is DEAE dextran.

Even more preferably said LHRH is the LHRH 2-10 form.

As used herein, the term "pharmaceutical" includes "veterinary".

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption or delayed release of the

injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying, the freeze-drying technique and the spray-drying technique which yield a powder of the active ingredients plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or compressed into tablets, or incorporated directly with the food of the diet. For oral administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1μg and 2000 μg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: A binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; and a lubricant such as magnesium stearate. When the dosage unit form is a capsule, it may

contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. A syrup or elixir may contain the active compound, methyl and propylparabens as preservatives, and a dye. Of course, any material used in preparing any dosage unit form should be veterinarily pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for veterinarily active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. For administration to livestock it is particularly advantageous to use a multi-dose container linked to a repeating vaccination gun. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5 μg to about 2000 μg. Expressed in proportions, the

active compound is generally present in from about 0.5 μg to about 2000 μg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

Although not intending to limit the invention to any one theory or mode of action, the induction of an effective immune response against LHRH results in prevention of the release of the hormones LH and FSH from the anterior pituitary. Since these hormones are required for the development and maintenance of the gonads, reduction in the levels of these hormones leads to a decrease or loss of reproductive functions. The vaccinated animals are therefore effectively neutered resulting in the loss of characteristics associated with sexual maturity such as fighting, wandering, sexual behaviour, loss of condition, organoleptic characteristics, tumours of reproductive organs and pregnancy.

Accordingly, another aspect of the present invention relates to a method of eliciting, in an animal, an effective immune response to LHRH said method comprising administering to said animal an effective amount of LHRH-conjugate.

Reference to "LHRH-conjugate" should be understood as a reference to the LHRH preparation of the present invention.

Reference to "animal" should be understood as the reference to all animals including primates (e.g. humans, monkeys), livestock animals (e.g. sheep, cows, horses, donkeys, goats, pigs), laboratory test animals (e.g. rats, guinea pigs, rabbits, hamsters), companion animals (e.g. dogs, cats), captive wild animals (e.g. emus, kangaroos, deer, foxes), aves (e.g. chickens, ducks, bantams, pheasants, emus, ostriches), reptiles (e.g. lizards, snakes, frogs) and fish (e.g. trout, salmon). Said animal may be male or female.

In a most preferred embodiment, the present invention relates to a method of eliciting, in an animal, an effective immune response to LHRH said method comprising administering to said

animal an effective amount of LHRH-conjugate wherein said immune response inhibits the reproductive capacity of said animal.

Preferably said LHRH-conjugate is LHRH 2-10 form.

Reference to "inhibiting the reproductive capacity of an animal" should be understood as the partial or complete reduction of the functioning of any one or more of the reproductive organs's activities or modulation of said animal's hormonal levels such that reproductive activity, such as sexual activity, is reduced.

Inhibiting the reproductive capacity of an animal may result in a number of consequences such as, but not limited to, the castration of said animal or the reduction or elimination of characteristics associated with sexual maturity (for example, fighting, wandering, sexual behaviour, loss of condition, unwanted organoleptic characteristics, tumours of reproductive organs and pregnancy). "Castration" should be understood as a reference to the neutering of both male and female animals. Inhibiting the reproductive capacity of an animal may also result in the cessation of tumor cell proliferation (for e.g. prostate cancer cells, breast cancer cells, ovarian cancer cells or testicular cancer cells), inhibition or reversal of hyperplasia, such as prostate enlargement, endometriosis or inflammatory responses.

Accordingly, another aspect of the present invention relates to a method of castrating an animal said method comprising administering to said animal an effective amount of LHRH-conjugate.

Preferably said LHRH-conjugate is the LHRH 2-10 form.

Yet another aspect of the present invention relates to a method of regulating oestrus cycling in a female animal said method comprising administering to said animal an effective amount of LHRH-conjugate.

Preferably said LHRH-conjugate is the LHRH 2-10 form.

Reference to "regulating" should be understood in its broadest sense and includes, for example, inhibiting or delaying oestrus.

Still yet another aspect of the present invention relates to a method of inhibiting characteristics induced by the sexual maturation of an animal said method comprising administering to said animal an effective amount of LHRH-conjugate.

Preferably said LHRH-conjugate is the LHRH 2-10 form.

Reference to "inhibiting characteristics induced by the sexual maturation of an animal" should be understood as a reference to the reduction or complete elimination of any one or more physical and/or behavioural characteristics induced either directly or indirectly by sexual maturation. Said physical and/or behavioural characteristics include, for example, fighting, wandering, sexual behaviour, loss of condition, unwanted organoleptic characteristics, oestrus cycling, fertility, pregnancy and tumours of the reproductive organs. Accordingly, inhibiting said characteristics includes inhibiting sexual activity (for example preventing female cattle mounting other female cattle) preventing or delaying ovulation, reducing aggressive behaviour or reducing unwanted organoleptic characteristics such as boar taint. In a particularly preferred embodiment, said characteristics are aggression and sexual activity.

According to this preferred embodiment there is provided a method of inhibiting aggression in an animal said method comprising administering to said animal an effective amount of LHRH 2-10 form-conjugate.

In another most preferred embodiment there is provided a method of inhibiting sexual activity in an animal said method comprising administering to said animal an effective amount of LHRH 2-10 form-conjugate.

Vaccination with LHRH conjugate in male dogs and cats can be used to control unwanted behaviour, particularly aggression and the urge to roam. In female dogs and cats, the desired effects are control of fertility and of unwanted behaviour at the time of oestrus, commonly termed "in heat" or "in season". The unwanted behaviour in females includes increased fractiousness, marking of territories, wandering and other behaviours associated with oestrus.

According to this most preferred embodiment there is provided a method of inhibiting characteristics induced by the sexual maturation of cats and/or dogs said method comprising administering to said cat and/or dog an effective amount of LHRH-conjugate.

Most preferably said characteristics are aggression and roaming in male cats and/or dogs and fractiousness, marking of territory, wandering and oestrus behaviour in female cats and/or dogs.

In the thoroughbred horse industry, the racing of stallions is associated with difficulty in handling and ease and consistency of training. A large proportion of young colts are gelded and raised as castrates to make them more manageable. This does not appear to impact significantly on their racing potential. A vaccine to control unwanted behavioural problems would allow the full racing potential of male horses to be realised, with the added benefit of reversibility and so obtaining the genetic benefit as a stud animal after their racing career is over.

The racing of fillies and mares (female horses) is at its height in the spring and to some degree in the autumn in the temperate climates of the world. It is at these times of the year that horses come into season. This causes difficulties in training, handling and in uneven and poor racing performance. An LHRH vaccine to control oestrus would have a large and ready market in the horse racing industry. There are currently products based on hormone analogues available to control oestrus in racing fillies and mares. These are reported to have a lasting effect on the ability of treated mares to breed.

Accordingly, in yet another preferred embodiment there is provided a method of inhibiting characteristics induced by the sexual maturation of horses said method comprising administering to said horse an effective amount of LHRH-conjugate.

Most preferably said characteristics are aggression in colts and oestrus behaviour and uneven performance in mares.

In cattle, the unmanageable behaviour of bulls is well known. Aggression of bulls can be directed toward stockmen, inanimate objects such as fences and drinking troughs and can result in serious fighting between cattle. Thus in most beef producing countries, bulls destined for beef production are castrated while still calves, and the resulting steers are raised. The raising of steers in preference to entire males has a significant negative impact on production performance, but this is judged to be an acceptable, even necessary trade off over the raising of more docile steers.

Heifers are raised for beef production in the USA and in Australia. The cycling of heifers in feedlots causes significant production losses. The cycling heifer has a large increase in activity levels, resulting in poor or negative growth over the 5-7 days of the cycle. The heightened activity levels of heifers in oestrus impacts on other heifers in the same pen, so that the production performance of the entire pen of 50-100 animals is affected. In the USA heifers are fed a diet containing melengestrol acetate (MGA), a synthetic steroid, to control oestrus. In Australia, and other countries where hormonal feed supplements are prohibited, heifers are raised in feedlots without feeding of MGA, with poor production performance.

Accordingly, the immunocastration of livestock, although reducing or eliminating characteristics associated with sexual maturity, generally results in a negative impact on production gains on immunocastrated animals over uncastrated animals. This theory is based on the well established fact that entire animals grow considerably faster and more efficiently than castrated animals. However, the inventor has determined that administering the LHRH preparation of the present invention to livestock nevertheless results in the achievement of

production gains. Reference herein to "production gains" includes but is not limited to an increase in final weight of livestock at slaughter, lowering of feed requirements for each kilogram of carcass weight gained, increasing growth rate of said livestock as compared to uncastrated livestock, improving the quality of meat derived from said livestock (for example, by controlling unwanted organoleptic characteristics of said meat) or decreasing stress levels of intensively housed livestock by reducing aggressive interactions of the intensively housed animals or, with respect to pigs, control of boar taint.

Accordingly, yet another aspect of the present invention relates to a method of achieving production gains in livestock said method comprising administering to said livestock an effective amount of LHRH-conjugate.

Preferably said production gain is the reduction or elimination of unwanted organoleptic characteristics of meat from male livestock.

The LHRH-conjugate may be administered to the livestock in a single-dose, for example a single administration of a slow or pulsatile release vaccine or in multiple doses.

Preferably said LHRH-conjugate is the LHRH 2-10 form.

Accordingly, there is provided a method of achieving production gains in livestock said method comprising administering to said livestock an effective amount of an LHRH 2-10 form-conjugate.

Preferably said production gain is the reduction or elimination of unwanted organoleptic characteristics of meat from male livestock.

The term "livestock" includes but is not limited to mammals such as pigs, cattle, sheep; captive wild animals such as deer; and aves such as emus or ostriches. Most preferably, said livestock are pigs and cattle.

According to this most preferred embodiment, there is provided a method of achieving production gains in pigs said method comprising administering to said pigs an effective amount of an LHRH 2-10 form-conjugate.

Preferably said production gain is the reduction or elimination of boar taint.

In another most preferred embodiment, there is provided a method of achieving production gains in cattle said method comprising administering to said cattle an effective amount of an LHRH 2-10 form-conjugate.

In animals, and particularly humans, vaccination with LHRH-conjugate can be used as a prophylactic or therapeutic treatment for disorders which are modulated directly or in directly by LHRH. These disorders include malignancies of cells which are regulated by LHRH or regulated by hormones which are themselves regulated by LHRH, for example, testicular cancer, breast cancer, ovarian cancer, prostate cancer and cancers of oncofoetal cells or cells which are induced to express oncofoetal antigens when malignancy occurs. These disorders also include non malignant proliferative disorders such as hyperplasias, for example, prostatic hyperplasia or endometrial hyperplasia. Without limiting the present invention to any one theory or mode of action, some tumor cells express receptors for reproductive hormones, the synthesis of which are regulated by LHRH. By vaccinating against LHRH it is possible to prevent the release of these hormones. The LHRH-conjugate of the present invention may also be used to treat or prevent disorders such as ovarian polycystitis, endometriosis and inflammatory conditions. Further uses of the LHRH-conjugate of the present invention include human fertility treatment based on modulation of the libido.

Accordingly, another aspect of the present invention relates to a method of inhibiting the growth of cells which are regulated directly or indirectly by LHRH said method comprising administering an effective amount of LHRH-conjugate.

Preferably said cells are human cells.

Reference to cell "growth" is a reference to the proliferation, differentiation or functional activity of said cell. Reference to cell growth which is "regulated directly or indirectly by LHRH" should be understood as a reference to cell growth which is regulated by LHRH itself or cell growth which is regulated by hormones other than LHRH which are themselves either directly or indirectly regulated by LHRH.

Reference to "inhibiting" should be understood as a reference to the prevention of cell growth, the cessation of cell growth or the down regulation of cell growth. Said cells may be located within the organ from which they derive or at some other location within the animal's body, such as, for example, where a malignant breast cell has metastasised in the liver.

In a particularly preferred embodiment said cells are malignant cells and most particularly malignant testicular cells, malignant breast cells, malignant ovarian cells or malignant prostate cells.

In yet another preferred embodiment said cells are hyperplastic cells such as prostatic hyperplastic cells or endometrial hyperplastic cells.

In yet another aspect of the present invention there is provided a method of down-regulating the libido of an animal said method comprising administering to said animal an effective amount of LHRH-conjugate.

Preferably said animal is a human.

Further features of the present invention are more fully described in the following Examples. It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention. It should not be understood in any way as a restriction on the broad description of the invention as set out above.

EXAMPLE 1

PREPARATION OF LHRH-CONJUGATE COMPOSITION

The LHRH-conjugate is based on a synthetic 2-10 form of Lutenising Hormone Releasing Hormone (LHRH) peptide coupled to a carrier protein. The peptide by itself is too small to be antigenic, and coupling to a carrier protein is required so that the peptide acts as a hapten and immunity is induced to LHRH. The carrier protein is diphtheria-toxoid.

The peptide is synthesised by Fmoc chemistry and the resulting 2-10 form LHRH peptide is coupled to diphtheria toxoid. The coupling may be performed as described in Ladd et al. 1990 or in Bonneau et al. 1994, and the resulting conjugate of peptide and diphtheria-toxoid processed to be free of unbound peptide and other by-products of conjugation. Such processing may be achieved by conventional dialysis or by ultrafiltration.

The resulting LHRH-carrier preparation may be used to prepare a composition for administration by formulation with or in an adjuvant (referred to as "LHRH-conjugate"). The adjuvant is an ionic polysaccharide such as DEAE-dextran, dextran sulphate or QAE-dextran. The adjuvant formulation may include a combination of two or more of the adjuvants listed. These lists are not to be taken as exhaustive. The selection of adjuvant is in part dependant on the species being targeted and is based on the level and duration of the immune response required and on the lack of reactogenicity (ie tissue compatibility). The level of active component and adjuvant are chosen to achieve the desired level and duration of immune response.

Formulations of LHRH-conjugate suitable for use in the present invention are preferably in the range of 5-500μg of LHRH-diphtheria toxoid in 5-500mg of DEAE-dextran.

EXAMPLE 2

The LHRH vaccine (2-10 LHRH with diphtheria toxoid and DEAE dextran adjuvant as described in Example 1) was given to pigs as:

Group 1: 2 doses at 14 and 18 weeks of age, 10 male pigs per group.

Group 2: 3 doses at 14, 18 and 22 weeks of age, 10 male pigs per group.

Group 3: Controls received DEAE-dextran adjuvant alone, 10 male pigs per group.

Doses of LHRH vaccine were given subcutaneously. The LHRH vaccine is preferably in the range of 50-500μg of LHRH-diphtheria toxoid in 50-500mg of DEAE-dextran.

Pigs were slaughtered at 22 weeks of age (groups 1 and 3) or at 24 weeks (group 2).

Parameters measured:

Anti-LHRH titres were measured at 2 weeks post 2nd dose.

Boat taint compounds skatole and androstenone were measured in fat samples take at slaughter.

Results:

Anti-LHRH titres 2 weeks post boost. Group mean titres are shown.

Group	Titre
Group 1	4300
Group 2	2760
Group 3	<20

* Boar taint compounds were measured in samples taken at slaughter.

Group mean values are shown.

Group	Skatole*	Androstenone*
Group 1	0.06	0.18
Group 2	0.05	0.23
Group 3	0.07	0.51

* Boar taint compounds are expressed as μg/g fat tissue.

Conclusions:

The LHRH vaccine induced high levels of antibody in all vaccinated pigs as determined at 2 weeks post boost.

The LHRH vaccine was able to control boar taint compounds in all vaccinated pigs.

EXAMPLE 3

MICE

10 mice were vaccinated with formulations consisting of analogues of LHRH, linked to diphtheria toxoid and adjuvanted with DEAE dextran. Mice were vaccinated on days 0 and 14 and bled on day 21 to demonstrate induction of antibody to LHRH.

Analogues of LHRH tested in mice include:

2-10 LHRH: His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2. (SEQ ID NO:2)

Modified 2-10: Gly-Ser-Gly-Ser-Gly-Leu-Arg-Pro-Gly-NH2. (SEQ ID NO:4)

Both constructs were linked to diphtheria toxoid by conventional chemistries. Mice received between 5-50μg of conjugate per injection in 5-50mg DEAE dextran adjuvant.

LHRH-conjugate	titre 1 week post boost
2-10 - diphtheria toxoid	3005 (n = 9)
Modified 2-10	1990 (n = 7)

Titres to LHRH were induced in all mice vaccinated with the above constructs.

EXAMPLE 4

CATTLE

Entire male and female cattle were vaccinated with a formulation comprising 2-10 LHRH conjugated to diphtheria toxoid, and adjuvanted with DEAE dextran. Cattle were 9-12 months of age at the time of initial vaccination. Each dose contained between 50 and 500µg conjugate formulated in DEAE-dextran (50-500mg).

Vaccinations were at 0 days with a boost vaccination at 28 days. Blood samples were taken at monthly intervals after the boost vaccination, and antibody titres measured by ELISA.

Female cattle (heifers) behaviour was monitored by daily inspection by trained farm staff and veterinarians. 8 weeks after boost vaccination, behaviour was also monitored by fixing of Heat Mount Detector pads (Kamar Marketing Group Inc, Steamboat Springs, Colorado, USA, dye releasing pads) to the rump of heifers. Mounting or riding behaviour (also called bulling) by cycling heifers will crush capsules of dye in the pads, which can be visualised from a distance. This usually only occurs when the standing heifer is receptive, ie in oestrus, and when the mounting heifer is also in oestrus. Thus the pads provide a useful continual monitor of oestrus in vaccinated heifers run with control unvaccinated heifers.

Results:

Anti-LHRH titres.

GMT = Geometric mean titre of group

Vaccination Group	GMT 3 weeks post boost (range)	GMT 7 weeks post boost (range)
Placebo vaccinated controls	<100	<100
LHRH-diphtheria toxoid conjugate in DEAE dextran adjuvant	10,357 (3623-26133)	3435 (1538-15464)

Riding behaviour/Pad reactivity:

Control heifers (female cattle) exhibited riding behaviour at the time of primary and boost vaccination and during the first 7 weeks after boost vaccination. None of the vaccinated cattle exhibited the behavioural patterns associated with reproductive function in cycling heifers. Scoring of cycling behaviour by direct observations were confirmed by Heat Mount Detector Pads, none of which were activated in vaccinates during the 7 week post boost period.

These results confirm the ability of the preferred formulation vaccine to modify behaviour of vaccinated animals, in this example the control of oestrus and associated behaviours in female cattle (heifers).

EXAMPLE 5

DOGS

Beagle/Foxhound cross dogs and bitches were vaccinated with a formulation comprising 2-10 LHRH conjugated to diphtheria toxoid, and adjuvanted with DEAE dextran. Dogs were 6 -10 months of age at the time of initial vaccination. Control dogs were not vaccinated.

Vaccinations were at 0 days with a boost vaccination at 28 days. Blood samples were taken at

monthly intervals after the boost vaccination, and antibody titres measured by ELISA.

The dose of vaccine is preferably in the range of 50-500μg LHRH-diphtheria toxoid in 10-100mg DEAE-dextran.

Titres to LHRH in dog serum:

Weeks post boost vaccination	Vaccinated with LHRH vaccine	Unvaccinated controls
0	<100	<100
4	84,640	<100
8	38,919	<100
12	7,900	<100

All titres shown are Geometric Mean Titres of the group of 7-8 dogs. Titres were measured by ELISA.

The data show that the favoured formulation of 2-10 LHRH conjugated to diphtheria toxoid in DEAE-dextran adjuvant induces a strong antibody response in 100% of vaccinated dogs. Of significance in this example is the demonstration that the preferred formulation is able to give duration of the antibody response.

Inhibition of development of testes:

At 16 weeks post boost, testes sizes were measured in controls and vaccinates, by reference to orchidometer beads.

Testes size (Group Mean Values):

Weeks post boost vaccination	Vaccinated with LHRH vaccine	Unvaccinated controls
16	0.5 cm^3	12 cm^3

These data demonstrate that the preferred formulation is able to prevent the development of reproductive organs, as shown in this example in the inhibition of the growth and maintenance of testes in dogs.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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SEQUENCE LISTING

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(ii) TITLE OF INVENTION: IMMUNOGENIC LHRH COMPOSITIONS AND METHODS RELATING THERETO

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Glu His Trp Ser Tyr Gly Leu Arg Pro Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

His Trp Ser Tyr Gly Leu Arg Pro Gly
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Trp Ser Tyr Gly Leu Arg Pro Gly
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Ser Gly Ser Gly Leu Arg Pro Gly
1 5

CLAIMS:

1. A composition for use in eliciting an effective immune response to LHRH said composition comprising a LHRH-diphtheria toxoid conjugate adsorbed to an ionic polysaccharide.

2. The composition according to claim 1 wherein said ionic polysaccharide is DEAE-dextran.

3. The composition according to claim 1 or 2 wherein said LHRH is LHRH 2-10 form.

4. The composition according to claim 1 or 2 wherein said LHRH is modified LHRH 2-10 form.

5. A pharmaceutical composition comprising a LHRH-diphtheria toxoid conjugate adsorbed to an ionic polysaccharide together with one or more pharmaceutically acceptable carriers and/or diluents.

6. The pharmaceutical composition according to claim 5 wherein said ionic polysaccharide is DEAE-dextran.

7. The pharmaceutical composition according to claims 5 or 6 wherein said LHRH is LHRH 2-10 form.

8. The pharmaceutical composition according to claims 5 or 6 wherein said LHRH is modified LHRH 2-10 form.

9. A method of eliciting, in an animal, an effective immune response to LHRH said method comprising administering to said animal an effective amount of the composition of any one of claims 1 to 8.

10. A method of eliciting, in an animal, an effective immune response to LHRH said method comprising administering to said animal an effective amount of the composition of any one of claims 1 to 8 wherein said immune response inhibits the reproductive capacity of said animal.

11. A method of castrating an animal said method comprising administering to said animal an effective amount of the composition of any one of claims 1 to 8.

12. A method of regulating oestrus cycling in a female animal said method comprising administering to said animal an effective amount of the composition of any one of claims 1 to 8.

13. A method of inhibiting characteristics induced by the sexual maturation of an animal said method comprising administering to said animal an effective amount of the composition of any one of claims 1 to 8.

14. The method according to claim 13 wherein said characteristic is aggression.

15. The method according to claim 13 wherein said characteristic is sexual activity.

16. The method according to claim 13 wherein said animal is a male cat and /or dog.

17. The method according to claim 16 wherein said characteristic is aggression and/or roaming.

18. The method according to claim 13 wherein said animal is a female cat and/or dog.

19. The method according to claim 18 wherein said characteristic is fractiousness, marking of territory, wandering and/or oestrus behaviour.

20. The method according to claim 13 wherein said animal is a male horse.

21. The method according to claim 20 wherein said characteristic is aggression.

22. The method according to claim 13 wherein said animal is a female horse.

23. The method according to claim 22 wherein said characteristic is oestrus behaviour and/or uneven performance.

24. A method of achieving production gains in livestock said method comprising administering to said livestock an effective amount of the composition of any one of claims 1 to 8.

25. The method according to claim 24 wherein said production gain is the reduction or elimination of unwanted organoleptic characteristics from the meat of said livestock.

26. The method according to claim 25 wherein said livestock are cattle, pigs, goats and/or sheep.

27. The method according to claim 25 wherein said livestock are pigs and said production gain is the reduction or elimination of boar taint.

28. The method according to claim 24 wherein said livestock are pigs.

29. The method according to claim 24 wherein said livestock are cattle.

30. A method of inhibiting the growth of cells which are regulated directly or indirectly by LHRH said method comprising administering an effective amount of the composition of any one of claims 1-8.

31. The method according to claim 30 wherein said cells are human cells.

32. The method according to claim 30 wherein said cells are malignant testicular cells, malignant breast cells, malignant prostate cells, malignant ovarian cells or malignant oncofoetal cells.

33. The method according to claim 30 wherein said cells are hyperplastic cells

34. The method according to claim 33 wherein said hyperplastic cells are prostate cells or endometrial cells.

35. A method of down-regulating the libido of an animal said method comprising administering to said animal an effective amount of the composition of any one of claims 1-8.

36. The method according to claim 35 wherein said animal is a human.

37. Use of the composition of any one of claims 1 to 8 to elicit, in an animal, an effective immune response to LHRH.

38. Use of the composition of any one of claims 1 to 8 to castrate an animal.

39. Use of the composition of any one of claims 1 to 8 to regulate the oestrus cycling of a female animal.

40. Use of the composition of any one of claims 1 to 8 to inhibit characteristics induced by the sexual maturation of an animal.

41. Use according to claim 40 wherein said characteristic is aggression.

42. Use according to claim 40 wherein said characteristic is sexual activity.

43. Use according to claim 40 wherein said animal is a cat and/or dog.

44. Use according to claim 43 wherein said characteristic is aggression, roaming, fractiousness, marking of territory, wandering and/or oestrus behaviour.

45. Use according to claim 40 wherein said animal is a horse.

46. Use according to claim 45 wherein said characteristic is aggression and/or uneven performance.

47. Use of the composition of any one of claims 1-8 to elicit production gains in livestock.

48. Use according to claim 47 wherein said production gain is the reduction or elimination of unwanted organoleptic characteristics from the meat of said livestock.

49. Use according to claim 48 wherein said livestock are cattle, pigs, goats and/or sheep.

50. Use according to claim 47 wherein said livestock are pigs and said production gain is the reduction or elimination of boar taint.

51. Use according to claim 47 wherein said livestock are pigs.

52. Use according to claim 47 wherein said livestock are cattle.

53. Use of the composition of any one of claims 1-8 to inhibit the growth of cells which are regulated either directly or indirectly by LHRH.

54. Use according to claim 53 wherein said cell are human cells.

55. Use according to claim 54 wherein said cells are malignant testicular cells, malignant breast cells, malignant prostate cells, malignant ovarian cells or malignant oncofoetal cells.

56. Use according to claim 54 wherein said cells are hyperplastic cells.

57. Use according to claim 56 wherein said hyperplastic cells are prostate cells or endometrial cells.

58. Use of the composition of any one of claims 1-8 to down-regulate the libido of an animal.

59. Use according to claim 58 wherein said animal is a human.

60. Use of a composition comprising a LHRH-diphtheria toxoid conjugate adsorbed to an ionic polysaccharide in the manufacture of a medicament for eliciting an effective immune response to LHRH.

61. Use of a composition according to claim 60 wherein said ionic polysaccharide is DEAE-dextran.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00532

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A61K 38/24, 39/39, 47/36, 47/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: A61K and keywords below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Derwent, Chemical Abstracts. Keywords: Luteinising hormone releasing hormone, gonadotropin releasing hormone, diphtheria, vaccine, dextran

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 88/05308 A (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 28 July 1988 See whole document	1-61
A	US 5378688 (NETT, T M et al) 3 January 1995 See whole document	1-61
A	GB 2228262 A (NATIONAL INSTITUTE OF IMMUNOLOGY) 22 August 1990 See whole document	1-61

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 August 1998	21 AUG 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **BERNARD NUTT** Telephone No.: (02) 6283 2491

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internati nal Application No.
PCT/AU 98/00532

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	88/05308	AU	11017/88	ZA	8800149		
US	5378688	AU	51860/90	WO	90/09799	ZA	9001391
GB	2228262	NONE					

END OF ANNEX